The role of PKB in the cytokine signalling of haemopoietic cells

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The Role of PKB in the Cytokine Signalling of Haemopoietic Cells

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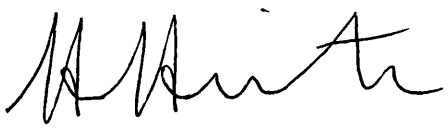

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Summary

Haemopoietic cells are dependent on the appropriate cytokine for their growth and survival. Upon cytokine withdrawal, haemopoietic cells cease to proliferate and undergo apoptosis. Treatment of responsive cells (BaF/3, FD-6 and MC9) with a number of cytokines, results in the activation of classI_A PI3K, which has been implicated in the proliferation and survival of many cell types. One downstream effector of PI3K, thought to be involved in growth and survival, is the serine/threonine kinase, PKB. Therefore, we investigated the role of PKB in the cytokine-mediated growth and survival of haemopoietic cells.

Treatment of responsive cells with IL-3, IL-4, GM-CSF, SCF and insulin resulted in the rapid phosphorylation and activation of PKB, which was inhibited by treatment with the PI3K inhibitor LY294002. However, the cytokine-mediated activation of PKB did not correlate with the growth and survival of haemopoietic cells in all cases. Nor was the activation of PKB sufficient to induce the hyper-phosphorylation of the pro-apoptotic Bcl-2 family member Bad in all cases. These results suggest that PKB activation alone is not sufficient to provide a survival and proliferative signal to these cells.

The major effect of PI3K inhibition by LY294002 observed in these cells was a reduction in the cytokine-mediated proliferation. LY294002 treatment did not result in a significant increase in apoptosis. To determine whether PKB is involved in this PI3K-mediated proliferation, the constitutively active PKB variant gagPKB was inducibly expressed in BaF/3 cells with the dominant negative PI3K, Δp85. Expression of Δp85 alone resulted in a substantial reduction in IL-3-stimulated cell growth and the co-expression of gagPKB in these cells did not reverse this inhibition. Therefore, other or additional pathways must be required for the mitogenic response of IL-3 that is mediated through PI3K.

In order to investigate in more detail the role of PKB in IL-3-mediated cell survival, PKB variants were expressed under the control of the tetracycline-regulated expression (tet off) promotor. The effects of the putative dominant negatives, cxPKB and kdPKB; wtPKB; and the constitutively active gagPKB on IL-3-mediated growth and survival were investigated. gagPKB expression did not increase the survival of these cells either in the presence or absence of IL-3. Interestingly kdPKB expression increased the phosphorylation of endogenous PKB, therefore, generating doubt about its effectiveness as a dominant negative. cxPKB expression did not significantly decrease the survival of these cells. These data question whether PKB is involved in the IL-3-mediated survival of BaF/3 cells.

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Chapter 1
Introduction

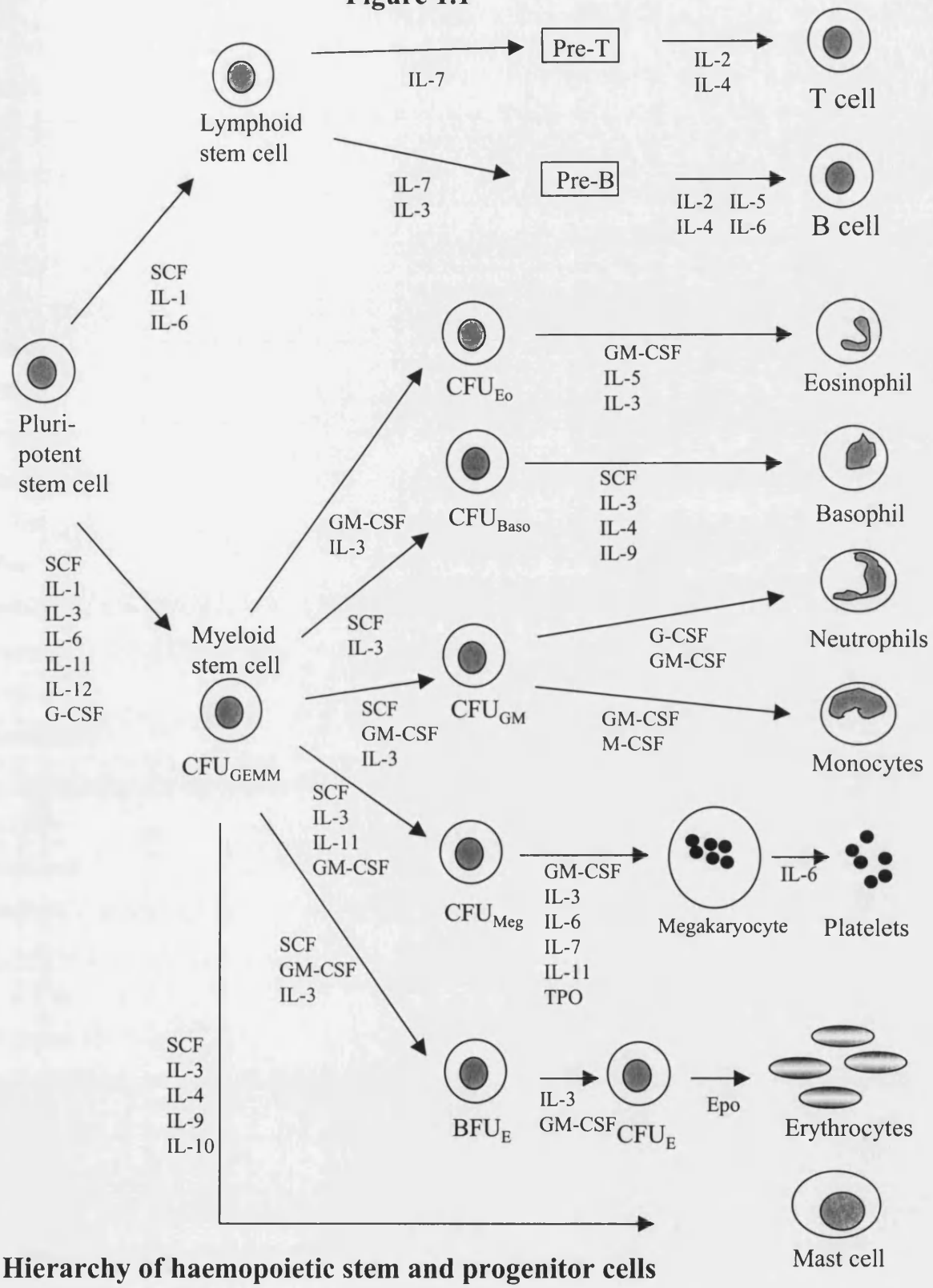
1.1 Cytokines and the Regulation of Haemopoiesis

All cells of the blood system are derived from a population of self-renewing multipotential haemopoietic stem cells located in the bone marrow (Metcalf, 1989). As the multipotential haemopoietic stem cells differentiate they become committed to specific lineages that go on to form myeloid and erythroid lines as well as the lymphoid lineages. The earliest progenitor precursors are the CLD (common lymphoid progenitor), which form the B- and T-cell lines, and the CMD (common myeloid progenitor), which give rise to granulocytes, erythroid, monocytes and megakaryocytes. More mature and specialised progenitors are termed CFU_{GM} (CFU= colony-forming unit in agar culture medium; GM= granulocytes and monocytes), CFU_{EO} (eosinophils), CFU_{Baso} (basophils), CFU_{Meg} (megakaryocytes), BFU_E (burst forming unit, erythroid) and CFU_E (erythroid) (Figure 1.1). These progenitor cells differentiate to form all the mature cells of the haemopoietic system (Figure 1.1) (Miyajima *et al.*, 1992).

Many haemopoietic cells have a high turnover rate. Therefore, to ensure that the correct balance between cell types is maintained, the survival, differentiation and proliferation of each is tightly regulated. The stem cell has the ability to self-renew, so although in adult mammals the bone marrow is a major site of new cell production, the overall levels of cells in the bone marrow remain constant. Progenitor cells must be able to maintain the correct balance of cell types under normal conditions and also be able to meet the requirements for increased and rapid production in infectious states or after blood loss. This tightly regulated process is termed haemopoiesis.

Haemopoiesis is regulated by cell-cell interactions and by a family of glycoproteins termed cytokines (Miyajima *et al.*, 1988; Arai *et al.*, 1990). These cytokine growth factors regulate haemopoiesis by controlling the differentiation, survival and proliferation of haemopoietic progenitor cells as well as mature cells. The actions of specific cytokines during the differentiation of haemopoietic cells are summarised in Figure 1.1.

Figure 1.1



Hierarchy of haemopoietic stem and progenitor cells

The stem cell gives rise to a common lymphoid stem cell and a myeloid precursor (CFU_{GEMM}) which gives rise to more mature and specialised progenitors: CFU_{GM} (granulocytes and monocytes), CFU_{Eo} (eosinophils), CFU_E (erythroid), CFU_{Baso} (basophils) and CFU_{Meg} (megakaryocytes). BFU_E (burst-forming unit, erythroid) refers to an earlier erythroid progenitor than the CFU_E (adapted from Miyajima *et al.*, 1992).

1.2 Cytokines and Their Receptors

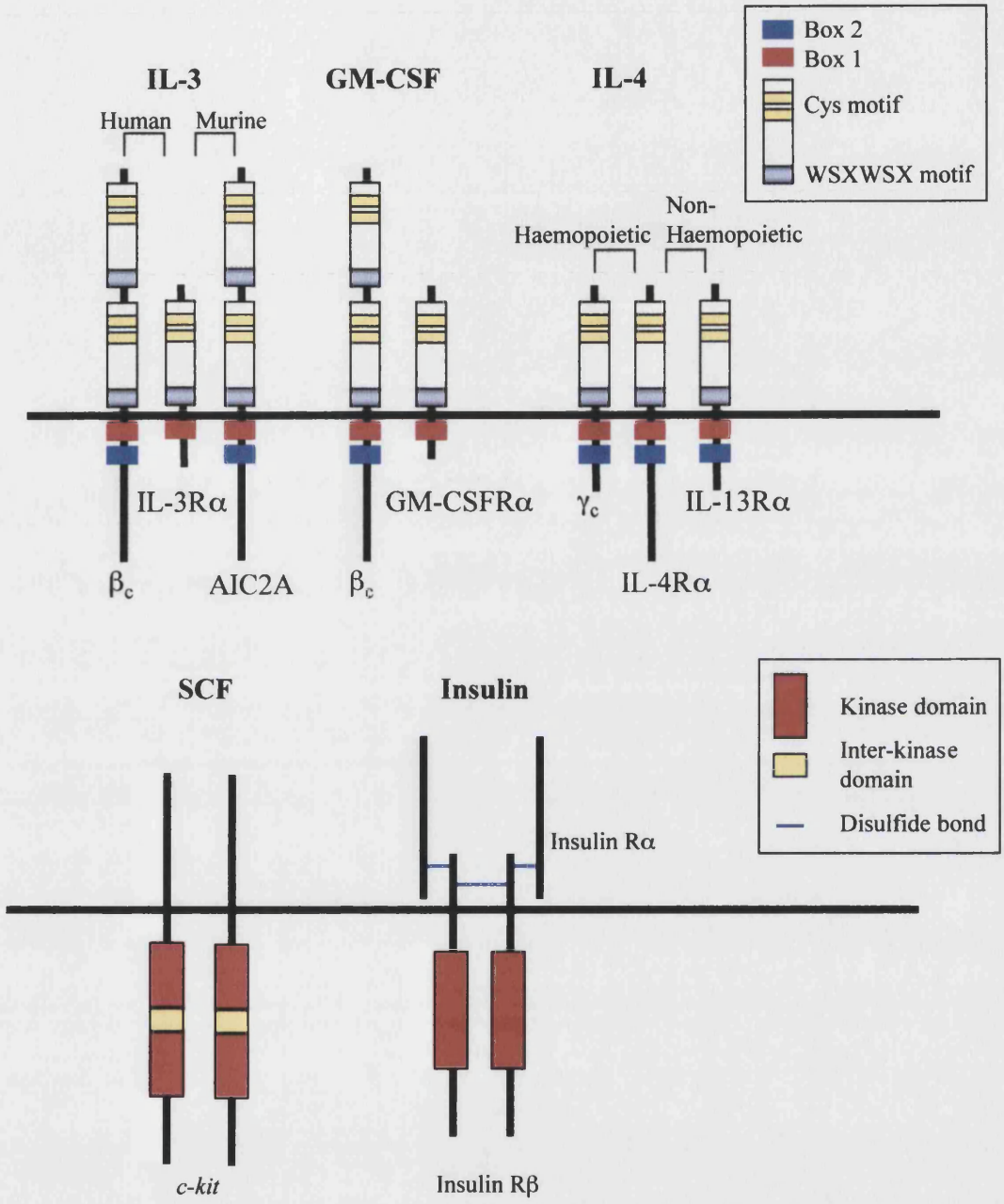
Haemopoeisis is controlled in part by a family of growth factors termed cytokines (cyto meaning cell and kine meaning movement). Most cytokines were either identified by their ability to promote the formation of colonies in semi-solid cultures of bone marrow cells and are therefore termed colony-stimulating factors (CSF's) (Metcalf, 1989) or by their actions on lymphocytes and are therefore termed interleukins (IL) (reviewed in Arai *et al.*, 1990). Cytokines are a diverse group of acidic glycoproteins with molecular mass ranging from 14-39 kDa. Little sequence homology exists between family members but analyses of their three-dimensional molecular structures indicate that they share many similar structural features (Bazan, 1990). Multiple cytokines have been identified and they include IL-1 to IL-20, stem cell factor (SCF), granulocyte-macrophage CSF, granulocyte CSF and erythropoietin.

The roles of cytokines in haemopoiesis are generally pleiotropic, influencing more than one cell type and many have overlapping activities. This means that specific cytokines can interact with more than one cell type and that the same effect on a particular haemopoietic cell lineage can be elicited by several cytokines (Arai *et al.*, 1990). The roles that the various cytokines play in each stage of development of the haemopoietic lines are summarised in Figure 1.1.

Cytokines elicit their effects by binding to discrete receptors that are expressed on the surface of responsive cells. The cloning of these receptors has revealed many similarities between the receptors and indeed many share receptor subunits. This may explain the functional pleiotropy and redundancy observed among several of the cytokines.

All the subunits of the IL-3R, GM-CSFR and IL-4R belong to the type I cytokine receptor family whose features include N-terminal $CX_{9-12}CXWX_{22-30}CX_{8-25}C$ (Cys motif) and WSXWS motifs, a single transmembrane domain and two regions of homology, termed the box 1 and box 2 regions, in the intracellular membrane proximal domain (Figure 1.2) (Cosman, 1993). Despite the cytokine-induced phosphorylation of the receptors and other cellular proteins in response to growth factor stimulation, the receptors in this family do not have intrinsic tyrosine kinase activity. Members of the type I cytokine receptor family utilise kinases of the Jak and Src families to transmit their signals through tyrosine phosphorylation pathways (Refer to Sections 1.2.3 and 1.2.4).

Figure 1.2
Structure of Receptor Complexes



The subunits which make up the receptor complexes for IL-3, IL-4, GM-CSF, SCF and insulin are shown. All the subunits of the receptors for IL-3, IL-4 and GM-CSF belong to the class I cytokine receptor family which do not have any intrinsic catalytic activity. In murine cells an additional IL-3-specific β subunit, AIC2A, exists. In haemopoietic cells IL-4 appears to signals through a γ_c/IL-4Rα complex, while in non-haemopoietic cells it signals through an IL-4Rα/IL-13Rα1 complex. The receptor for SCF, *c-kit,* and the insulinRβ subunit have intrinsic tyrosine kinase activity.

The receptors for SCF, PDGF and insulin belong to a family of receptors known as the protein tyrosine kinase receptor family. They are a family of more than 50 different transmembrane polypeptides with a tyrosine kinase domain in their intracellular portion. These receptor tyrosine kinases (RTK's), with the exception of insulin, consist of a single polypeptide chain with a large extracellular ligand-binding region, a single membrane spanning domain and large intracellular domain with intrinsic tyrosine kinase activity (Ullrich and Schlessinger, 1990). The structures of the receptor subunits are shown in Figure 1.5.

1.2.1 IL-3

Interleukin-3 (IL-3) is a 25kDa glycoprotein produced by activated T-lymphocytes and mast cells. It is involved in the early stages of development of multipotential progenitors and haemopoietic stem cells and can influence the development of macrophages, granulocytes, megakaryocytes, erythrocytes, eosinophils and mast cells. It also acts as a growth factor for these mature, differentiated cells (Arai *et al.*, 1990). Studies of IL-3 -/- mice showed that these mice are viable and no abnormalities in haemopoiesis were observed. However when these mice were infected with the parasite *Stronglyoides venezuelensis* fewer basophils and mast cells were produced than in wild type mice. This indicates that IL-3 is involved in immunity to parasites and one of its functions in host defence is to expand populations of haemopoietic effector cells (Lantz *et al.*, 1998).

IL-3 exerts its physiological effects by binding to its receptor on the surface of cells (Figure 1.2). The high affinity IL-3 receptor complex is composed of two distinct subunits α and β. IL-3Rα is a 70kDa glycoprotein that specifically binds IL-3 with low affinity. The β subunit is unable to bind IL-3 by itself, but together with IL-3Rα it forms a high affinity complex. IL-3Rβ, often referred to as β_{common} (β_c), also forms part of the receptor complexes for IL-5, and GM-CSF. In mice two β subunits exist: AIC2B is homologous to the human βc while AIC2A is IL-3 specific. This additional IL-3Rβ subunit is thought to have arisen by a gene duplication event (Miyajima *et al.*, 1992).

1.2.2 GM-CSF

GM-CSF is a 14-35kDa glycoprotein produced by endothelial cells and fibroblasts upon stimulation with TNF or IL-1; by macrophages activated with bacterial toxin or adherence; and by antigen-stimulated T cells. It acts on intermediate progenitor cells and in conjunction with other growth factors to support the differentiation of neutrophils, eosinophils, moncytes, erythrocytes and megakaryocytes (Demetri, 1992).

Like the IL-3 receptor complex GM-CSFR is composed of two subunits (Figure 1.2). The GM-CSF receptor complex uses the same β_c chain as the IL-3 (Miyajima *et al.*, 1992) and an 80kDa GM-CSFRα subunit. As with the IL-3R complex, β_c does not bind GM-CSF itself but together with the low affinity GM-CSFRα forms a high affinity receptor complex (Hayashida *et al.*, 1990).

1.2.3 IL-4

Interleukin-4 (IL-4) is a 20kDa glycoprotein produced by activated T-cells and mast cells. It acts on a number of haemopoietic cells e.g. B cells, T lymphocytes and mast cells, often in synergy with other cytokines. Other biological effects include induction of IgE class switching of B cells and expression of class II major histocompatibility complexes on resting B cells (Paul, 1991).

The receptor complex for IL-4 is composed of a high affinity 140kDa glycosylated α subunit (Mosley *et al.*, 1989) and a 64kDa γ_{common} (γ_c) subunit, which is shared with the receptors for IL-2, IL-7, IL-9 and IL-15 (Figure 1.2) (Russell *et al.*, 1993; Kondo *et al.*, 1993). Association of γ_c with IL-4Rα increases the IL-4 binding affinity 2-4 fold (Russell *et al.*, 1993). There is evidence to suggest that IL-4 and IL-13 also share a common receptor subunit. This is not the γ_c subunit (He and Malek, 1995), and in some haemopoietic cells the IL-4Rα subunit appears to be shared with IL-13 (Welham *et al.*, 1995; Smerzbertling and Duschl, 1995; Obiri *et al.*, 1997; Murata *et al.*, 1997). An additional 65-70kDa protein (IL-13Rα1) has been identified and appears to be a component of both receptor complexes in non-haemopoietic cells (Obiri *et al.*, 1997; Murata *et al.*, 1997).

1.2.4 SCF

Stem cell factor (SCF) is a 248 amino acid residue glycoprotein produced by stromal cells. It is the gene product of the *steel* locus, mutations of which cause defects in pigmentation, fertility and haemopoiesis in affected mice (Zsebo *et al.*, 1990; Flanagan and Leder, 1990). SCF is involved in the early events of the proliferation and differentiation of multipotential haemopoietic stem cells to various lineages. In particular, the proliferation of haemopoietic stem cells and the differentiation to mast and erythroid cell lineages (Williams *et al.*, 1992).

The receptor for SCF, *c-kit*, is a 160kDa glycoprotein (Figure 1.2) which belongs to a family of protein tyrosine kinase receptors whose members include the PDGF (platelet-derived growth factor) receptor and the CSF-1 (colony stimulatory factor-1) receptor, *c-fms*. *c-kit* is the gene product of the dominant spotting (W) locus, mutations in which display a similar phenotype to those seen when the *steel* locus is mutated (Chabot *et al.*, 1988). Upon ligand binding, *c-kit* dimerises, resulting in the activation of its intrinsic tyrosine kinase domains, autophosphorylation and signal transduction via tyrosine kinase pathways. Structural features shared with PDGF-R and *c-fms* include an extracellular region of 5 immunoglobulin loops and a cytoplasmic kinase region split by an interkinase domain (Qiu *et al.*, 1988).

1.2.5 Insulin

Insulin is a peptide growth factor that is composed of a 21 amino acid α and 30 amino acid β-chain. It is synthesised by the β cells of the pancreas and its major physiological role is the regulation of blood glucose levels. Insulin also acts as a survival and growth factor for some cells.

Insulin exerts its biological effects by binding to its receptor on the surface of responsive cells. The insulin receptor is a heterotetrameric structure comprising of two α and β subunits that are connected by disulfide bonds (Figure 1.2). The α subunit is extracellular and involved in ligand binding, while the β subunits traverse the plasma membrane. The intracellular region of the β-subunit contains an intrinsic tyrosine kinase. This activity is activated upon ligand binding and the subsequent receptor subunit heterotetramerisation (Yarden and Ullrich, 1988).

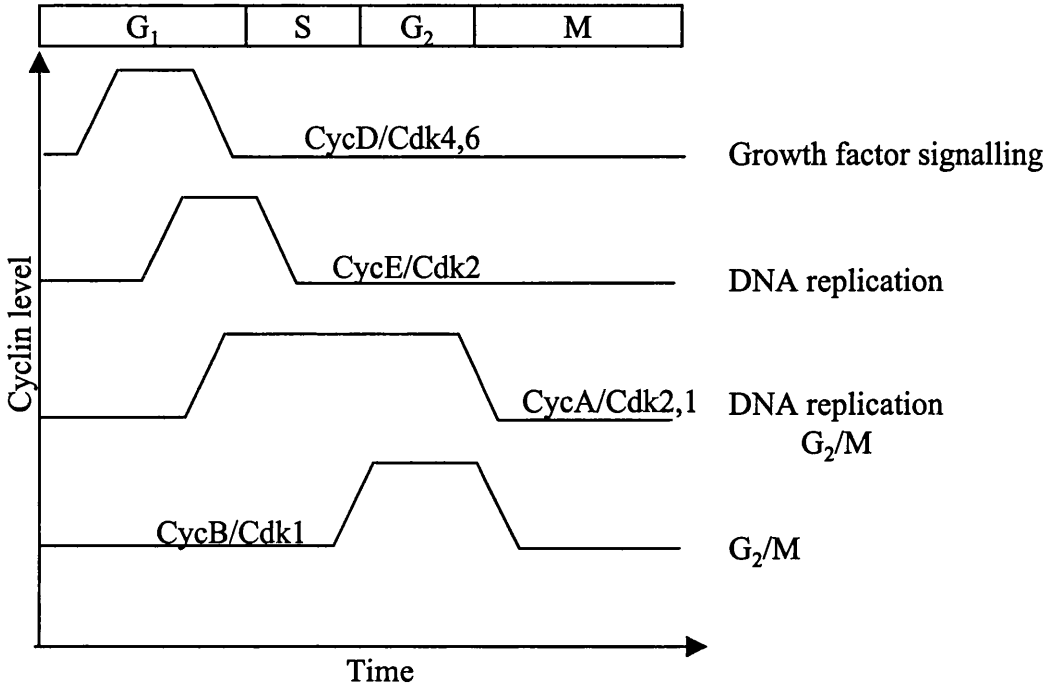
1.3 The Functions of Cytokines

1.3.1 Proliferation

Cytokines regulate haemopoiesis by controlling the differentiation, survival and proliferation of both progenitor and mature haemopoietic cells. During differentiation, when progenitor cells are irreversibly committed to one of the haemopoietic lineages, a massive expansion of progenitor cells also occurs. The haemopoietic system must also respond to acute situations such as infection or blood loss. This is accomplished by the rapid expansion and maturation of a specific set of haemopoietic cells at the affected site. This process of expansion, or proliferation, is stimulated by the actions of specific cytokines binding to their receptors on the surface of responsive cells and thus triggering the signalling pathways, which lead to cellular proliferation.

The proliferation of all eukaryotic cells, including those of the haemopoietic system, follows a specific program, which is known as the cell cycle. The cell cycle governs the progression of cells through their growth and their segregation into daughter cells. It also plays a pivotal role in determining whether a cell proliferates or withdraws into a quiescent state. The cell cycle can be divided into 4 successive phases: M phase, where mitosis occurs; G_1, which is the gap between M phase and S phase; S phase, where DNA synthesis occurs; and G_2, which is the gap between S phase and M phase. Before the cell can progress to the next phase of its cycle, regulatory mechanisms are in place to ensure that all the necessary events have taken place. These are termed checkpoints. For example, major checkpoints exist at the G_2/M phase interface to ensure that DNA synthesis has occurred correctly before mitosis, and at the G_1/S phase interface, which is sensitive to such things as DNA damage, growth factor withdrawal, contact inhibition and death receptor activation. A family of proteins termed cyclins regulate the progression of a cell through these checkpoints. These act as positive regulatory subunits to a family of cyclin-dependent protein kinases (CDKs). Specific cyclins bind to specific CDKs activating the complex at specific times during the cell cycle. CDKs are generally constitutively expressed while the expression of cyclins oscillates in respect to the cell cycle suggesting that the cyclins control the timing of the activation. Both the expression and degradation of cyclins are tightly regulated and dependent on the cell cycle. For example, several cyclins have been implicated in feedback and feedforward loops which regulate the transcription of other cyclins (Cross *et al.*, 1995).

Figure 1.3
Cyclin/Cdk Activity through the Cell Cycle



The Cdks associate with cyclin subunits at different stages of the cell cycle. D-type cyclins link G1 progression to growth control. Cyclin D levels remain high as long as growth factor is present in the media. Cyclins E, A and B oscillate. Cyclin E/Cdk2 and Cyclin A/Cdk2 seem to have a role in DNA replication. Cyclin A/Cdk1 and Cyclin B/Cdk1 promote the onset of mitosis. Adapted from Arello and Moreno 1997.

Table 1.1
E2F/RB-Family Complexes Through the Cell Cycle

RB Family Member	E2F Protein	Cell Cycle Stage
pRB	E2F-1, -2, -3, -4	G_0, G_1, S
P107	E2F-4	G_1, S, G_2
P130	E2F-4, -5	G_0, G_1

Summarised are the RB family members, the E2F proteins that they interact with and the stages of the cell cycle that these complexes are detected. Adapted from Dyson 1998.

Cyclins are subject to proteolytic degradation by the ubiquitin-dependent proteosome pathway and this also appears to be dependent on the cell cycle (Hochstrasser, 1995). The cyclin/CDK complexes and where they act in the cell cycle are summarised in Figure 1.3.

The activation of the cyclin/CDK complex is regulated both by phosphorylation and by its association with specific inhibitors. CDK activity is positively regulated by phosphorylation of Thr^{160} (in CDK2) by CDK-activating kinase (CAK), while the phosphoryation of Thr and Tyr residues near the N-terminus negatively regulates CDK activity (Morgan, 1995). Two major classes of CDK inhibitors have been identified in mammalian systems. The INK4 family specifically inhibit CDK4 and CDK6 thereby preventing cyclin D association. The Cip/Kip proteins bind to and inhibit a number of cyclin/CDK complexes. They can inhibit each of the cyclin/CDK complexes required for G_1 progression and entry into S phase.

Each of the cyclin/CDK complexes has a unique substrate specificity. One of the best characterised substrates of the cyclin/CDK complex is the retinoblastoma tumour suppressor pRB and the two other members of this family p107 and p130/RB2 (Hinds *et al.*, 1992). This family are sometimes referred to as pocket proteins because of their highly conserved pocket region that is necessary for the RB proteins to interact with other proteins and to repress transcription. RB proteins regulate cell cycle progression through their interactions with the E2F family of transcription factors. These interactions, and where they primarily occur in the cell cycle are summarised in Table 1.1.

When RB proteins are hypo-phosphorylated they interact with the E2F family of transcription factors and this inhibits the activity of the E2F proteins. For example, throughout G_1 phase the activity of the E2F transcription factors are repressed by the hypo-phophorylated pRB protein. Towards the end of G_1 pRB is phosphorylated by cyclin-D/CDK4/6 and cyclin-E/CDK2. This results in the release of free E2F, which in turn activates the transcription of cell cycle-regulated genes (Weinberg, 1995). One way in which pRB is thought to control E2F driven transcription is by acting as a bridge between E2F and histone deacetylase (HDAC1). HDAC1 mediates the removal of charged acetyl groups from core histones. This results in a tight association of DNA with the nucleosomes, which decreases the accessibility of transcription factors, in this case E2F, to promoters. The E2F family of transcription factors regulate the transcriptional activity of a number of genes that regulate cell cycle progression. These either encode functions that are important for DNA synthesis (for example DNA

polymeraseα (**295**) and thymidine kinase (Ogris *et al.*, 1993) or cell cycle regulators (including cyclin E (Botz *et al.*, 1996; Geng *et al.*, 1996), cyclin A (Schulze *et al.*, 1995) and cdk2). It is thought that the transcriptional activation of the cyclin genes by E2F creates a feed-forward loop, allowing amplification of the signals that drive the progression of the cell cycle. For example, at the end of G_1, the cyclin-E/CDK2 complex phosphorylates pRB, which results in the release of E2F. E2F can then upregulate the expression of cyclin-E, and this amplifies the signal to progress through to S phase.

1.3.2 Apoptosis

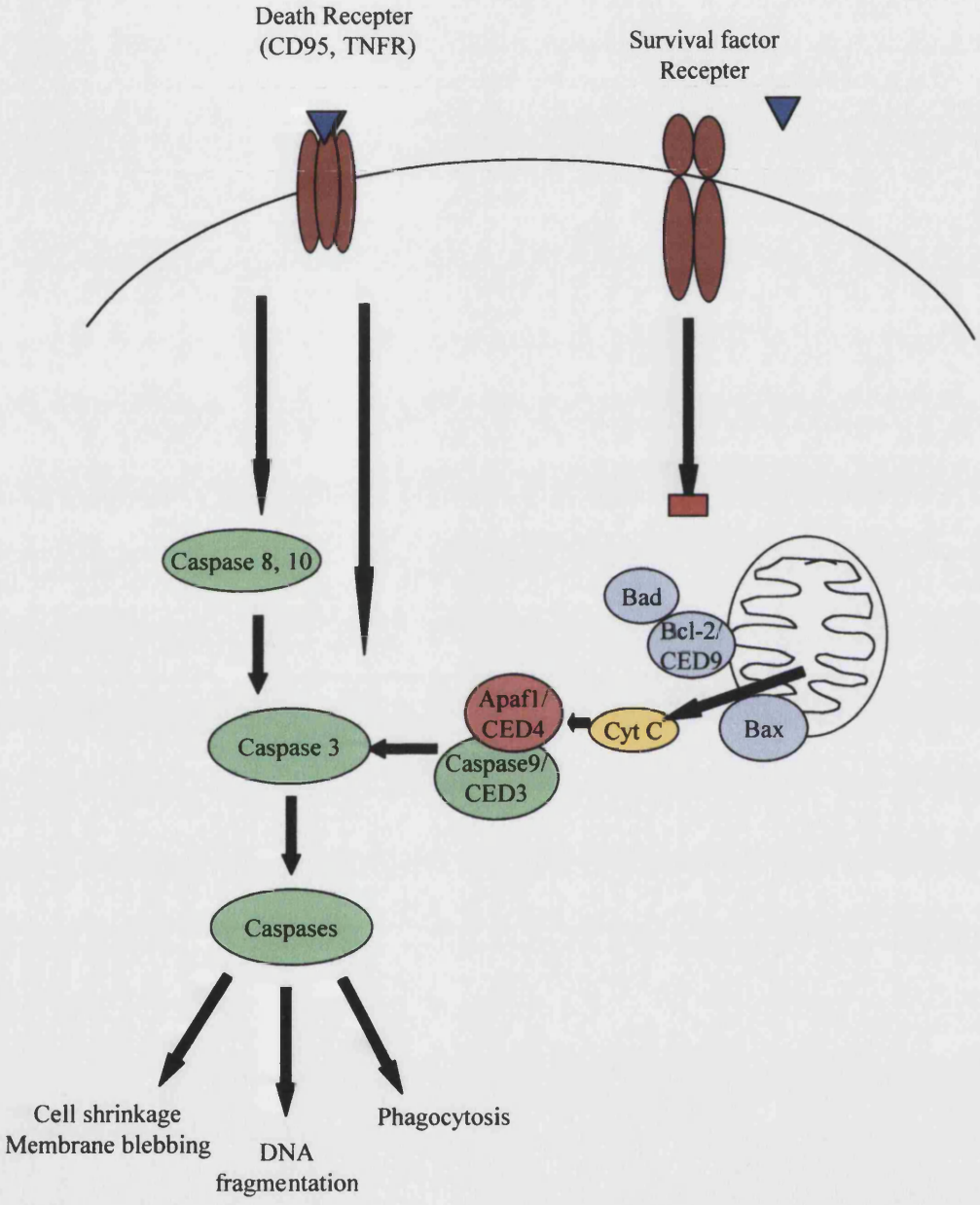
Prevention of cell death is also an important function of cytokines during haemopoiesis. Apoptosis (or programmed cell death) plays a crucial role in regulating cell numbers. It is an essential process not only in haemopoiesis but also for developing normal embryos, maintenance of homeostasis in all adult tissues and suppression of carcinogenesis. Apoptosis is the ordered disassembly of the cell from within. It is characterised by changes in the phospholipid content of the plasma membrane, membrane blebbing, cell shrinkage, mitochondrial changes, chromatin condensation and DNA fragmentation. The mitochondria are thought to be pivotal in controlling the apoptotic process. In the mitochondria of apoptotic cells electron transport and ATP production are disrupted, redox potential is lost and proteins that are involved in the downstream events of apoptosis (for example cytochrome c) are released. The process of apoptosis does not provoke an inflammatory response. Instead, phagocytic cells recognise the presence of phosphatidylserine on the outer leaflet of the plasma membrane and engulf the apoptotic cell.

Contrasting with apoptotic cell death is necrotic death, which is usually the result of severe cellular insult. Both internal organelle and plasma membrane integrity are lost, resulting in spilling of the cytosolic contents into the surrounding environment. Immune cells are attracted to the area and begin producing cytokines that generate an inflammatory response.

The apoptotic process can be triggered by a number of stimuli including growth factor withdrawal, death receptor activation, UV irradiation and stress. Broadly speaking these can be separated into two pathways: positive induction by death receptor engagement or negative induction by loss of suppressor activity (for example upon growth factor withdrawal).

Most of what we understand about the mechanisms of apoptosis has come from developmental studies in the nematode worm *Caenorhabditis elegans*. Studies of *C. elegans* mutations have identified three *C. elegans* gene products that are essential for apoptosis: CED-3 (where CED stands for cell death abnormal) and CED-4 promote apoptosis while CED-9 inhibits apoptosis. Mammalian homologues for these genes have been identified and CED-3 belongs to a family of proteases called cytosolic aspartate-specific proteases or caspases (Thornberry, 1999), CED-4 and its homologue Apaf-1 (apoptotic protease activating factor-1) are cytochrome-c binding adaptor proteins (Zou *et al.*, 1997) and CED-9 is an anti-apoptotic member of the Bcl-2 family (Vaux *et al.*, 1992). The roles that these proteins play in the apoptotic pathway are not fully understood but recently much progress has been made in understanding their functions. An overview of the roles that the Bcl-2 family and caspases play in apoptosis is shown in Figure 1.4 and they are discussed in more detail below.

Figure 1.4
The Roles of Bcl-2 family members and caspases in apoptosis



Apoptosis is a regulated physiological process, which can be induced by the withdrawal of a survival signal or by a specific signal to die. Caspases are central to the apoptotic process and once activated induce the cleavage of specific cytoskeletal and nuclear proteins leading to the characteristic cell shrinkage and DNA fragmentation of apoptotic cells.

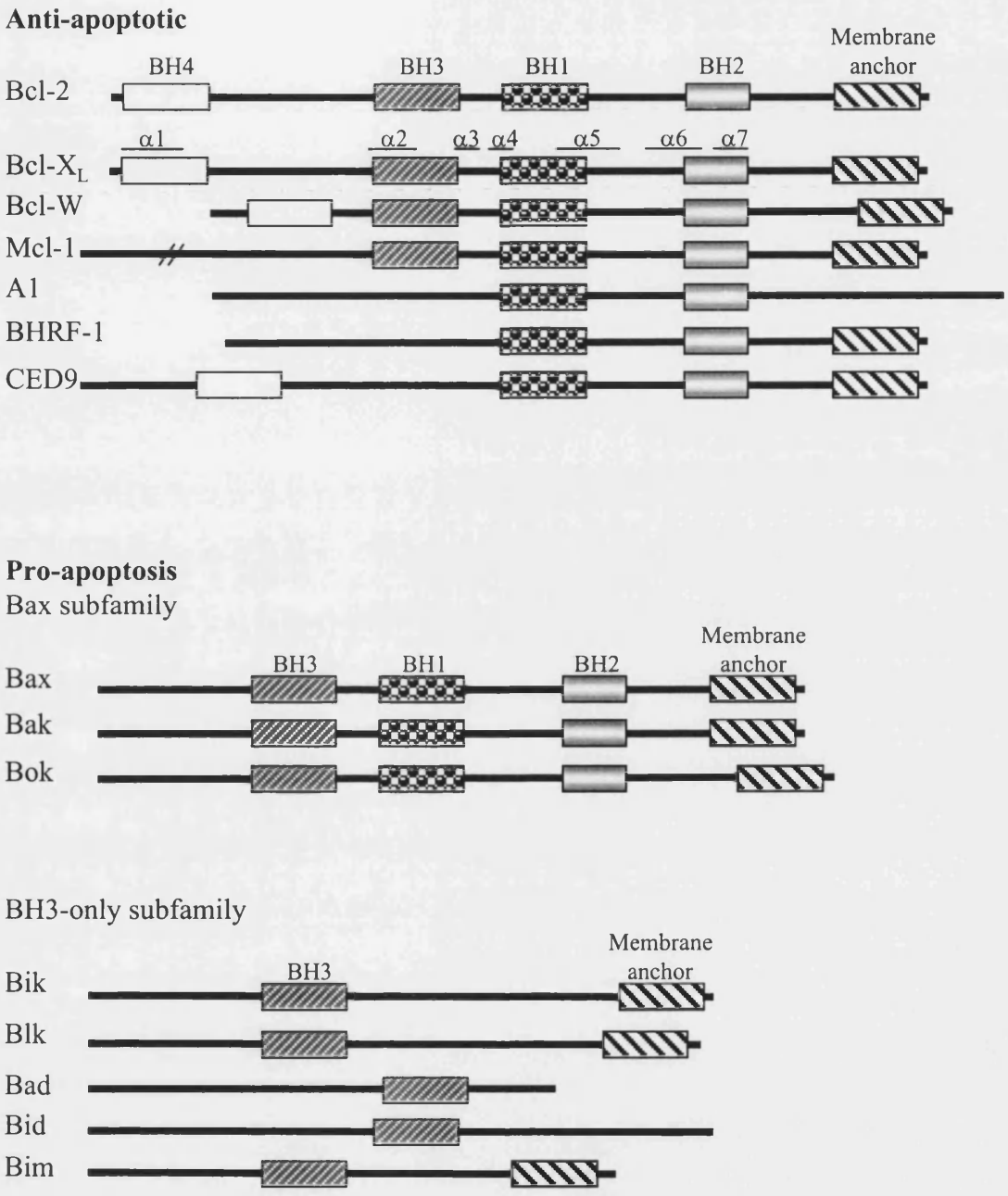
The Bcl-2 Family

The Bcl-2 protein family are intracellular regulators of cell survival. This family consists of both pro-apoptotic and anti-apoptotic members. The homology within the family is restricted to 4 Bcl-2 homology (BH) domains of which all members have at least one (Figure 1.4). Most anti-apoptotic members have at least BH1 and BH2 while the pro-apoptotic members can be further classified into those that consist of the BH3 domain only (eg Bad) and those that consist of BH1-BH3 domains (e.g. Bax). The pro- and anti-apoptotic family members can heterodimerise and seemingly titrate one anothers function, suggesting that their relative concentrations may act to control entry into the apoptotic pathway. The activity and interactive ability of some family members is also controlled by phosphorylation (Reed, 1997; Adams and Cory, 1998; Antonsson and Martinou, 2000).

Structural studies of the anti-apoptotic member Bcl-X_L show that the BH1, BH2 and BH3 domains form α-helices that create an elongated hydrophobic cleft to which an amphipathic BH3 α-helix can bind (Muchmore *et al.*, 1996). The overall structure of Bcl-X_L shows a remarkable similarity to membrane insertion domains of some bacterial toxins leading to the hypothesis that it is a membrane pore forming protein. In support of this theory, Bcl-2, Bcl-X_L, and Bax have all been shown to form ion channels in lipid bilayers *in vitro* (Schendel *et al.*, 1997; Antonsson *et al.*, 1997).

How the anti-apoptotic members of the Bcl-2 family act to prevent the action of caspases and CED-4/apaf-1 is not well understood. The BH4 domain of Bcl-2 is required for its interaction with CED-4 (Huang *et al.*, 1998), as well as its anti-apoptotic activity (Cheng *et al.*, 1997). Cytochrome-c is released from the mitochondria into the cytosol during many types of apoptosis (Rosse *et al.*, 1998; Bossy-Wetzel and Green, 1999). When in the cytosol, cytochrome-c forms a complex with Apaf1 and caspase-9 and this leads to the activation of caspase 9 (Li *et al.*, 1997). The pro-apoptotic Bcl-2 family member Bax can directly induce the release of cytochrome-c from the mitochondria (Jurgensmeier *et al.*, 1998; Eskes *et al.*, 1998), while expression of Bcl-2 prevents the release of cytochrome-c (Kluck *et al.*, 1997). Therefore, it seems likely that the Bcl-2 family regulate apoptosis at least in part by controlling the release of cytochrome-c from the mitochondria and its interaction with Apaf1 and caspase-9.

Figure 1.5
Structure of the Bcl-2 Family



The sub families of the Bcl-2 family. The anti-apoptotic members promote the survival of cells, while the pro-apoptotic members facilitate apoptosis. The Bcl homology (BH) domains of sequence homology, and membrane anchoring domains are indicated. The α-helices as determined by structural studies of Bcl-X_L are indicated α1-α7. This is not a comprehensive list and is adapted from Adams and Cory, 1998.

Caspases

Caspases are a family of highly specific cysteine proteases that have distinct roles in both inflammation and apoptosis. They are expressed as pro-enzymes that contain 3 domains: an N-terminal domain, a large subunit and a small subunit. Activation involves proteolitic processing between domains followed by association of the large and small subunits to form a heterodimer. Caspases are involved in apoptosis pathways resulting from both positive and negative induction. Caspase-8 activity is induced by the death receptors, while caspase-9 activity is induced by the dATP-dependent formation of a complex with Apaf1 and cytochrome-c upon the release of cytochrome-c from the mitochondria (Li *et al.*, 1997; Zhang *et al.*, 1993). Caspase 8 and 9 are referred to as initiator caspases. Activation of either of these initiator caspases leads to the activation of the effector caspase, caspase-3. This in turn can activate other effector caspases and thus amplify the apoptotic signal (Thornberry, 1999). Caspases contribute to the apoptotic process by the cleavage of various cellular proteins. A number of these are involved in the degradation of DNA, which is a hallmark of apoptosis. For example, targets of caspases include I^{CAD}, an inhibitor of the nuclease responsible for DNA fragmentation (Paradis and Ruvkun, 1998; Liu *et al.*, 1998) and PARP (poly (ADP-ribose) polymerase) (Demurcia and Demurcia, 1994; Lippke *et al.*, 1996). PARP is a DNA repair enzyme whose expression is triggered by DNA-strand breaks. In cells undergoing apoptosis PARP is cleaved and this is thought to facilitate the degradation of DNA. Other targets of caspases include focal adhesion kinase (FAK), which is involved in cytoskeleton regulation (Wen *et al.*, 1997) and Bcl-2. The BH4 domain of Bcl-2 is cleaved during apoptosis by a caspase action, and this accelerates the apoptotic process (Grandgirard *et al.*, 1998).

1.4 Signalling pathways activated by cytokines

Cytokines regulate haemopoiesis by modulating the differentiation, proliferation and survival of haemopoietic lineages and their precursors. These effects are mediated through the activation of several signalling pathways. Receptor binding leads to the rapid activation of a number of signalling proteins and pathways, most notably the src kinases, and the MAP kinase, Jak/STAT, and PI3K pathways. These will be reviewed in turn.

1.4.1 The Jak/STAT Pathway

Jaks are cytoplasmic tyrosine kinases that participate in signaling from a range of cell-surface receptors, particularly members of the cytokine family, which lack tyrosine kinase activity. Jak was originally an acronym for just another kinase, but has also been proposed as an acronym for Janus (the two-faced Roman God of gates and doorways) kinase. Four members of the Jak family have been identified: Jak1, Jak2, Jak3 (Witthuhn *et al.*, 1994) and Tyk2, which vary in size from 110kDa to 140kDa. Jak1, Jak2 and Tyk2 are all ubiquitously expressed, while Jak3 is predominantly found in myeloid cells, natural killer cells and T lymphocytes (Rane and Reddy, 1994; Johnston *et al.*, 1994).

Amino acid sequence alignment of the Jak family show that they possess seven highly homologous Jak homology domains (JH1-JH7) and have no protein interacting SH2 or SH3 domains (Figure 1.6A). The C-terminal region consists of a tandem kinase (JH1) and psuedo kinase (JH2) domain, only one of which (JH1) is catalytically active (Leonard and O'Shea, 1998).

All the cytokines whose receptors belong to the class I receptor family, which lack intrinsic kinase activity, induce the phosphorylation and activation of Jak family members. Stimulation with IL-4 leads to the activation of both Jak1 and Jak3 (Witthuhn *et al.*, 1994; Yin *et al*,. 1994) where as IL-3 (Silvennoinen *et al.*, 1993), GM-CSF (Quelle *et al.*, 1994) and SCF (Weiler *et al.*, 1996) stimulation results in the activation of Jak2.

Jaks are associated with the cytoplasmic domain of cytokine receptors but are catalytically inactive in resting cells. Studies using cytokine receptor mutants have shown that the membrane proximal box1 conserved motif is required for Jak association with receptor subunits (Quelle *et al.*, 1994; Tanner *et al.*, 1995). The Jak kinases bind to the receptor subunits through their N-terminal region. The JH6 and JH7 domains are necessary and sufficient for binding of Jak1 and 3 to γc (Zhou *et al.*, 1997) and Jak2 to

βc (Li *et al.*, 1997). Upon ligand binding, Jaks become rapidly activated by phosphorylation on tyrosine residue(s) within the activation loop of their kinase domain. It is believed that Jaks activate each other by cross-phosphorylation when they are brought into contact with each other through receptor homo- or hetero-dimerisation.

Cytokine-induced activation of the Jak family of kinases results in the tyrosine phosphorylation of a number of cellular proteins including the cytokine receptors themselves and a family of proteins known as STATs (signal transducers and activators of transcription).

STATs are latent cytoplasmic transcription factors that upon activation translocate to the nucleus where they regulate gene expression. Seven members of this family have been identified, STAT1-6 (including STAT5a and 5b), which vary in size from 734-851 amino acids. They are composed of a central DNA binding domain and an SH2 domain in the C-terminal region (Figure 1.6B). A conserved tyrosine residue exists near the C-terminus and its phosphorylation by Jak leads to STAT activation (Leonard and O'Shea, 1998).

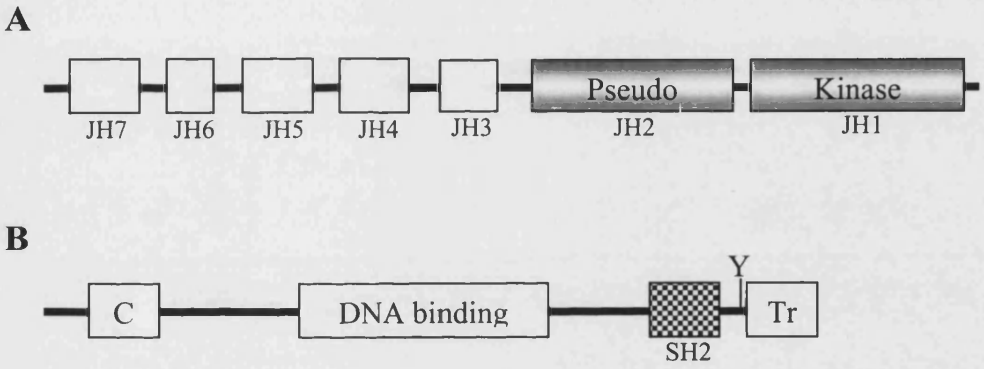
STAT proteins are recruited to the active cytokine receptor complex through their SH2 domain, where they either interact directly with phospho-tyrosine residues on the receptor or indirectly through other STATs or Jaks. The SH2 domain seems to direct the specificity of cytokine-induced STAT activation. STAT6 is activated by IL-4 treatment and has been shown to bind through its SH2 domain to IL-4Rα (Hou *et al.*, 1994). IL-3 and GM-CSF have been shown to activate both STAT5a and STAT5b, which interact with βc through their SH2 domains (Mui *et al.*, 1995). Interestingly, SCF has been reported to induce the phosphorylation and activation of STAT1α, STAT5a and STAT5b (Brizzi *et al.*, 1999). The mechanism of activation and whether Jak proteins are needed is unclear.

Once phosphorylated and activated by Jak, STAT proteins form homo- or hetero-dimers through the interaction of a phosphorylated tyrosine on one STAT molecule with the SH2 domain of another. The dimerised STAT proteins then translocate to the nucleus where they bind to DNA sequences most of which are related to the gamma interferon activated site (GAS), a regulatory element in the promoter of IFN-γ-inducible genes (Darnell *et al.*, 1994).

In addition to activating STATs, Jaks are also involved in the activation of a number of other proteins. Perhaps the most important function of the Jak family is the tyrosine phosphorylation of the class I cytokine receptors. Receptor phosphorylation is required for the recruitment (either directly or indirectly) and activation of a number of

signalling proteins to the receptor complex including the adaptor proteins Shc, Grb2, Gab2, the IRS family, the tyrosine phosphatases SHP1 and SHP2, and the lipid kinase PI3K.

Figure 1.6
Structure of Jaks and STATs



(A) The overall structure of Jak family members. The seven conserved domains, designated JH1 to JH7 are indicated. The C-terminal domain, JH 1 is the functional kinase domain, the pseudo-kinase domain, JH 2 lies immediately N-terminal to JH 1. **(B)** The functional domains of STATs are indicated, including a conserved region in the amino terminus (C), the DNA binding domain, the SH2 domain (SH2) and the C-terminal transcriptional activation domain (Tr). The conserved Tyrosine residue that is essential for STAT activation is shown.

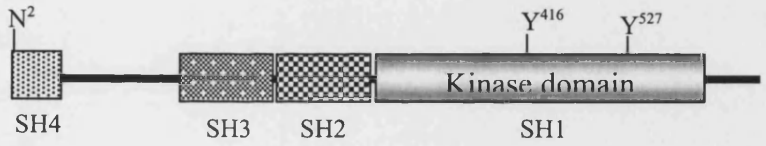
1.4.2 Src Kinases

The src family of tyrosine kinases were named after their first member to be identified v-src, the first characterised oncogene from the Rous sarcoma virus (Purchio *et al.*, 1978). Nine kinases of the Src family have now been identified: Src, Fyn, Yes, Fgr, Lyn, Hck, Lck, Yrk and Blk. They are characterised by four regions of homology referred to as src homology (SH) domains (Figure 1.7). SH1 is a C-terminal tyrosine kinase domain, SH4 an N-terminal membrane localisation sequence. Src homology regions 2 (SH2) and 3 (SH3) mediate the interactions of src kinases with other proteins. SH2 domains bind phospho-tyrosine residues in the motif YEEI, while SH3 domains interact with proline rich regions in a left handed helical conformation. Both the SH2 and SH3 domains are present in many other proteins where they are again involved in the interaction between proteins (Brown and Cooper, 1996).

Src kinases are activated in response to stimulation by a number of growth factors. The Src kinase family members Fyn, Lyn and Hck are activated in response to IL-3 stimulation (Anderson and Jorgensen, 1995), while Lyn (Linnekin *et al.*, 1997), Lck and Yes (Krystal *et al.*, 1998) have been shown to be activated in response to SCF.

Figure 1.7

Structure of c-Src Kinase



The overall structure of the Src family of protein kinases is shown. The regions of Src homology domains are indicated SH1 to SH4. SH1 is the kinase domain, SH2 is a region of protein-protein interactions, which binds phospho-tyrosine residues in the motif YEEI, SH3 domains interact with proline rich regions and the SH4 domain contains a myristylation motif. N^2, the site of myristylation, Y^{416}, which is the site of autophosphorylation and Y^{527}, the negative regulatory site are indicated.

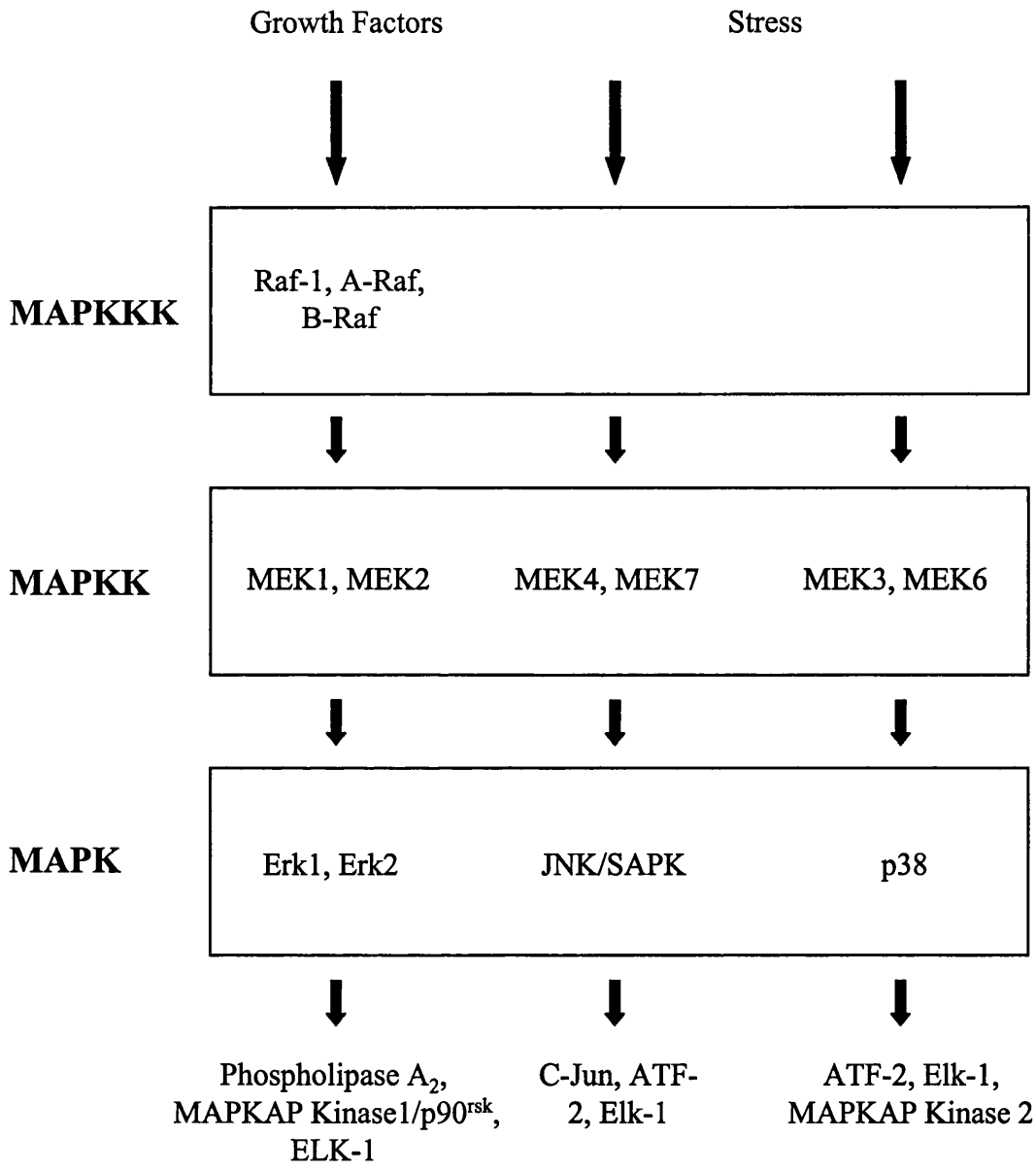
1.4.3 The MAP Kinase Pathway

The MAP (mitogen-activated protein) kinase cascades are characterized by their core three-component protein kinase cascade, which consists of a MAP kinase kinase kinase, which phosphorylates and activates a MAP kinase kinase, which in turn phosphorylates and activates MAP kinases (Figure 1.8). The MAP kinases are proline directed serine/threonine kinases that are characterized by their activation through the dual phosphorylation of tyrosine and threonine residues. The classical example of a MAP Kinase pathway is the Ras/Raf/MEK/ERK pathway, which results in the activation of the extracellular regulated kinases-1 and -2 (ERK-1 and -2). Two other MAK kinase cascades leading to the activation of c-Jun amino-terminal kinase (JNK) and p38 MAP kinase also exist.

The ERK Pathway

The classical MAP kinase pathway, which results in the activation of Erk-1 and -2, is activated in response to growth factor and cytokine stimulation through the activation of Ras. The Ras proteins are 21kDa plasma membrane associated proteins, which bind guanine nucleotides and have intrinsic GTPase activity. Three isoforms have been identified to date: c-H-*ras*, c-K-*ras* and c-N-*ras*. They were first identified as the transforming agents of the Harvey and Kirsten sarcoma viruses (Harvey, 1964; Kirsten and Mayer, 1967). Ras proteins act as regulatory switches whose activity is controlled by cycling between an active guanosine triphosphate (GTP)-bound and an inactive guanosine diphosphate (GDP)-bound state (Figure 1.9) (Polakais and McCormick, 1993). Ras bound GDP is exchanged for GTP, which is hydrolysed by Ras to GDP. The exchange of GDP for GTP is regulated by guanine nucleotide exchange factors (eg sos and Ras-GRF), which accelerate the exchange of Ras-GDP for Ras-GTP and hence activate Ras (Feig, 1994). It is thought that upon growth factor receptor engagement Ras is activated primarily by the translocation of Sos to the plasma membrane where Ras is located. Sos interacts with the adaptor protein Grb2 (Rozakis-Adcock *et al.*, 1993; Egan *et al.*, 1993; Li *et al.*, 1994), which can interact with activated growth factor receptors either directly (Lowenstein *et al.*, 1992; Rozakis-Adcock *et al.*, 1993; Buday and Downward, 1993; Skolnik *et al.*, 1993) or through the adaptor protein Shc (Ruff-Jamison *et al.*, 1993; Welham *et al*, 1994a; Salcini *et al.*, 1994) or the phosphatase/adaptor SHP2 (Welham *et al.*, 1994b; Tauchi *et al.*, 1994; Tauchi *et al.*, 1995).

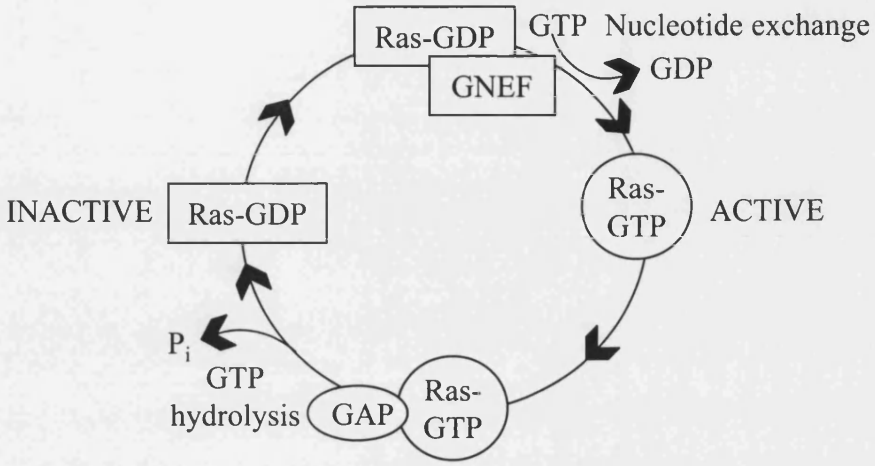
Figure 1.8
Mitogen-Activated Protein Kinase Cascades



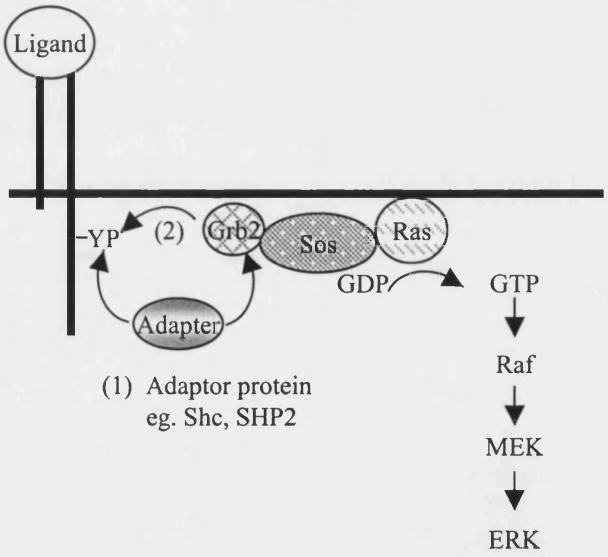
The MAP kinase cascades leading to the activation of the MAP kinases Erk, JNK and p38 are illustrated schematically. Growth factor activation lead to the activation of the Erk kinases by MEK1 and 2. Cellular stress results in the activation of the JNK and p38 MAPKs through MKK4/7 and MKK3/6 respectively. Once activated the MAPKs can transcription factors (Elk-1, c-Jun, ATF-2), other kinases (MAPKAP kinase1 and 2) and other regulatory enzymes (PLA_2). These downstream targets control cellular responses including growth, survival and differentiation.

Figure 1.9

(A) Cycling of p21^{ras} between GTP and GDP bound states



(B) Activation of Ras/MAPK pathway by activated receptors



(A) In its inactive state, Ras is bound to GDP. Guanine nucleotide exchange factors (GNEF) accelerate the exchange of GDP for GTP, activating Ras. GTPase activating proteins (GAPs) increase the the intrinsic GTPase activity of Ras, converting the active GTP-bound Ras, back to the inactive GDP-bound state. **(B)** Ras activity is regulated through the translocation of the Grb2-Sos complex to the membrane. This may occur (1) through the direct association of Grb2 with the activated receptor or (2) through the association of an adapter protein with the activated receptor.

The activation of Ras leads to the activation of the MAP kinase kinase kinase Raf-1. Raf is a ubiquitously expressed 72-76kDa cytoplasmic serine/threonine kinase, which was first identified as the cellular counterpart of the oncogenic *v-raf* from the murine sarcoma virus 3611 (Rapp *et al.*, 1983). Two other mammalian Raf family members have been identified: A-Raf (Beck *et al.*, 1987) and B-Raf (Eychene *et al.*, 1992).

Expression of a dominant inhibitory Raf-1 or Raf-1 anti-sense mRNA inhibits v-*ras*-induced transformation of fibroblasts indicating that Raf-1 functions downstream of Ras (Kolch *et al.*, 1991). Using the yeast two-hybrid system and *in vitro* binding studies it has been shown that Raf-1 can directly interact with Ras and this interaction is dependent on Ras being in its GTP-bound active state (Vanaelst *et al.*, 1993; Vojtek *et al.*, 1993; Zhang *et al.*, 1993). However, this direct association does not lead to Raf-1 activation, rather the recruitment of Raf-1 to the membrane is needed for activation but is not sufficient for the stable activation of Raf-1 (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). The observation that Raf-1 is hyper-phosphorylated in response to many signalling events has suggested that phosphorylation may play a role in regulating Raf-1 activity (Morrison *et al.*, 1993). Phosphorylation could directly modulate Raf-1 kinase activity or it could act by mediating protein-protein interactions. Phosphorylation on Ser^{338} and Tyr^{341} synergize to activate Raf-1 (Mason *et al.*, 1999), but the phosphorylation of Raf-1 on other sites, also appears to regulate Raf-1 activity. Raf-1 interacts with the adaptor protein 14-3-3 through phosphorylated Ser^{259} and Ser^{621} (Tzivion *et al.*, 1998) and this appears to positively regulate Raf-1 as over-expression of 14-3-3 in fibroblasts resulted in Raf-1 activation (Li *et al.*, 1995). However the mechanism by which 14-3-3 regulates Raf-1 is poorly understood.

The MAP kinase kinase, MEK (Map kinase/Erk activating kinase) is activated by the phosphorylation of residues Ser^{217} or Ser^{221} by Raf-1 (Alessi *et al.*, 1994). Two mammalian MEK isoforms have been identified: MEK1 and MEK2 (Crews and Erikson, 1992; Zheng and Guan, 1993). The activity of MEK is thought to be enhanced by MP1 (MEK partner 1) binding (Schaeffer *et al.*, 1998). The MEK kinases catalyse the phosphorylation of the MAP kinases, Erk1 and Erk2, on Thr^{183} and Tyr^{185} (Payne *et al.*, 1991). Upon growth factor stimulation, a proportion of the erk kinases are rapidly translocated from the cytoplasm to the nucleus (Lenormand *et al.*, 1993). MAP kinases are proline-directed kinases that phosphorylate the consensus sequence Pro-X-Ser/Thr-Pro (Gonzalez *et al.*, 1991). Their substrates include downstream kinases (eg.phospholipase A (Lin *et al.*, 1993) and the ribosomal S6 kinase $p90^{rsk}$ (Sturgill *et al.*, 1988), transcription factors (eg. Elk-1, which is involved in c-fos induction (Marais *et*

al., 1993) and the protein kinases that lead to the activation of Erks. Erk1 has been shown to phosphorylate MEK1 on Thr^{386}, which has been suggested to act as a negative feedback control reducing MEK1 activity (Brunet *et al.*, 1994). Raf1 is also phosphorylated by the Erk kinases and again this phosphorylation has been suggested to play a negative regulatory role (Anderson *et al.*, 1991; Lee *et al.*, 1992).

Ras (Duronio *et al.*, 1992) and the MAP kinases (Erk-1 and Erk-2) (Welham *et al.*, 1992), have been shown to be activated in response to IL-3, GM-CSF, and SCF. However, IL-4 is does not induce the activation of Ras nor the Erk kinases (Duronio *et al.*, 1992; Welham *et al.*, 1992).

The p38 Pathway

The MAP kinase pathway leading to the activation of the p38 kinase is activated in response to cellular stresses such as changes in osmolarity, DNA damage, UV irradiation and heat-shock as well as by the pro-inflammatory cytokines TNFα and IL-1. p38 is activated by the MAP kinase kinases MKK3 and MKK6, which catalyse the dual phosphorylation of p38 on the motif Thr-Gly-Tyr. Activated p38 subsequently phosphorylates and activates the transcription factors ATF-2 and Elk-1 and the MAP kinase activated protein kinase-2 (MAPKAP kinase-2) (Herlaar and Brown, 1999).

The JNK Pathway

The JNK kinases, also known as the stress-activated protein kinases (SAPKs) are activated in response to environmental stress and inflammatory cytokines. They are activated by the dual phosphorylation within the motif Thr-Pro-Tyr by the MAP kinase kinases MKK4 and MKK7. Activation of the JNK kinases results in the activation of the transcription factors c-Jun, ATF-2 and Elk-1 (Ip and Davis, 1998).

1.5 Phosphoinositide-3 Kinases (PI3K)

PI3K was first discovered through the observation that a PI kinase activity associated with the oncogenic $pp60^{v\text{-}src}$ (Sugimoto *et al.*, 1985). At first this was thought to be an intrinsic catalytic activity, but it was subsequently discovered that the PI kinase activity could be separated from the middle T $pp60^{c\text{-}src}$ complex by a high salt and detergent solution (Kaplan *et al.*, 1986; Whitman *et al.*, 1987). This PI kinase activity was found to catalyse the phosphorylation of the D3 position of the inositol ring thus generating a previously unknown class of lipids (Whitman *et al.*, 1988b). Interest in this new class of lipid kinase grew when it was discovered that all the mutants of the polyoma middle T that failed to associate with PI3K also failed to transform 3T3 fibroblasts (Kaplan *et al.*, 1986; Courtneidge and Heber, 1987; Whitman *et al.*, 1988a). This suggested a role for PI3K in oncogenic and mitogenic signal transduction.

PI3Ks phosphorylate the hydroxyl group at the 3' position on the inositol ring of phosphatidyl inositols. Phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PI(4)P) and phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) are converted to phosphatidylinositol-3-phosphate (PI(3)P), phosphatidylinositol-3,4-bisphosphate ($PI(3,4)P_2$) and phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$) respectively. PI(3)P is constitutively present in cells and its levels are largely unchanged by stimulation with a variety of ligands. In contrast $PI(3,4)P_2$ and $PI(3,4,5)P_3$ are almost absent from resting cells and are produced upon cellular stimulation. This makes $PI(3,4)P_2$ and $PI(3,4,5)P_3$ likely second messengers. Unlike the hydrolysis of $PI(4,5)P_2$ by phospholipase-C to IP_3 and diacylglyceraol, the lipid products of PI3K are not substrates for phospholipases, instead their 3 or 5 phosphate groups are removed by phosphatases such as PTEN and SHIP (Section 1.9).

Numerous PI3K isoforms exist and these can be divided into 3 classes based on their substrates and the adaptor proteins with which they interact (Table 1.2).

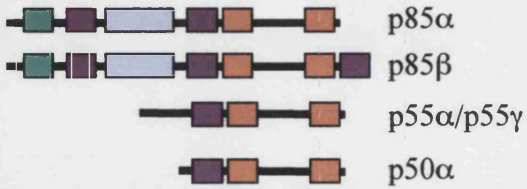
3.5.1 Class I (refer to Section 1.6 for more detail)

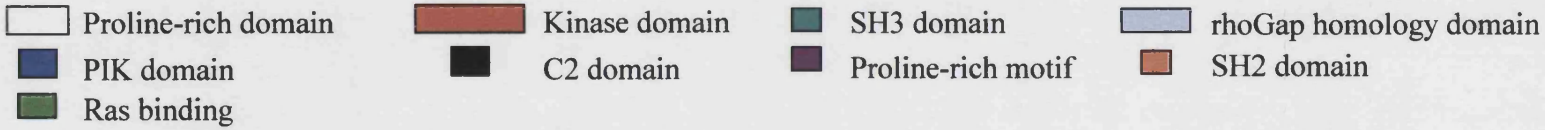
These PI3Ks phosphorylate all the above phospholipids in *in vitro* assays, although their preferred substrate is thought to be $PI(4,5)P_2$. Members of this class interact with active GTP-bound Ras and form heterodimers with adaptor proteins. They can be divided into two types (Class I_A and I_B) according to the nature of the interactions with adaptor proteins (Reviewed in Vanhaesebroeck *et al.*, 1997, Wymann and Pirola, 1998, Leevers *et al.*, 1999, Vanhaesebroeck and Alessi, 2000). Class I_A PI3Ks catalytic subunits vary in size from 110-130kDa and interact with SH2 domain containing adaptor proteins. They include the mammalian p110α, β and δ isoforms that interact with p85 adaptor subunits. Class I_B interact with stimulated G-protein βγ subunits including p110γ. A regulatory p101 subunit has been found which interacts with this subunit (Stephens *et al.*, 1997).

3.5.2 Class II

This class of PI3K are larger (>170kDa), and *in vitro* prefer PI and PI(4)P over $PI(4,5)P_2$ as substrates (Macdougall *et al.*, 1995; Domin *et al.*, 1997). They are characterised by a synaptotagmin C2 domain in their C-terminal region. C2 domains are involved in the regulation of synaptotagmin by Ca^{2+}, however, in the class II PI3Ks this domain is mutated at a critical Asp residue and Ca^{2+} is not required for phospholipid binding (Macdougall *et al.*, 1995; Arcaro *et al.*, 1998). As with the class I PI3Ks, class II PI3Ks are activated in response to extracellular signals. *In vitro* kinase assays have demonstrated that class II PI3Ks are activated in response to growth factors such as insulin (Brown *et al.*, 1999), EGF (Arcaro *et al.*, 1998), PDGF (Arcaro *et al.*, 1998), and integrins (Zhang *et al.*, 1995) and chemokines (Turner *et al.*, 1998). However, fractionation studies have shown that in contrast to class I PI3Ks, class II PI3Ks are primarily membrane associated (Arcaro *et al.*, 1998; Domin *et al.*, 1997). Recently, it has been reported that clathrin functions as an adaptor for the class II PI3K, PI3K C2α, binding to its N-terminal and stimulating its catalytic activity (Gaidarov *et al.*, 2001). Furthermore, exogenous expression of PI3K-C2α was found to affect clathrin-mediated endocytosis and sorting of the trans-Golgi network, suggesting a role for class II PI3Ks in membrane trafficking (Gaidarov *et al.*, 2001).

Table 1.2
Classification of PI3K Family Members

Class	Catalytic Subunit	Substrate Specificity	Adapter Binding Partner
I_A		PI PI(4)P $PI(4,5)P_2$	
I_B			p101
II		PI PI(4)P	Clathrin
III		PI	Vsp15p/p150



The classes of PI3K and the defining features of their subunits are shown. The assignment of the catalytic subunits to each class is based on their structural features, substrate specificity and the adapter subunits with which they associate.

3.5.3 Class III

A single class III catalytic subunit has been identified in all eukaryotic species, which exists as a complex with a serine/threonine kinase adaptor molecule (p150 in mammals). PI3K and its adaptor p150 are homologous to the yeast vps34p (vacuolar protein sorting 34p) and vps15p respectively. Vps15p is myristolated at its N-terminus and this targets the PI3K complex to the membrane (Panaretou *et al.*, 1997). Vsp34p is involved in the sorting of carboxypeptidase from the late golgi to the intermediate endosomal compartment, suggesting a role for class III PI3Ks in the sorting of proteins to the yeast vacuole (Schu *et al.*, 1993). Class III PI3Ks phosphorylate only PI *in vitro* to produce PI3P (Schu *et al.*, 1993). Levels of PI3P remain relatively constant in the cell, which suggests that the physiological processes that are controlled by this class of PI3Ks are constitutive and not activated in response to cellular stimulation.

1.5.4 Class I_A PI3K

Three mammalian class I_A PI3K have been identified to date. p110α (Hiles *et al.*, 1992) and p110β (Hu *et al.*, 1993) appear to be ubiquitously expressed while p110δ (Vanhaesebroeck *et al.*, 1997) is found mainly in haemopoietic cells. The p110 subunits are 110kDa proteins that interact with the p85 adaptor subunits through amino acids 20-108 of their N-terminal domain (Dhand *et al.*, 1994b). They contain a Ras binding domain and a C-terminal lipid kinase core. In addition to their lipid kinase activity, class I_A PI 3K also possess serine/threonine protein kinase activity. p85 is a substrate of the kinase activity of p110α and phosphorylation of p85 on Ser^{608} results in a marked decrease in kinase activity of PI3K (Dhand *et al.*, 1994a; Carpenter *et al.*, 1993). Interestingly p110δ is unable to phosphorylate p85 but undergoes autophosphorylation which inhibits its lipid kinase activity (Vanhaesebroeck *et al.*, 1997; Vanhaesebroeck *et al.*, 1999). A schematic diagram of the p110 PI3K subunit is shown in Table 1.2.

There are two mammalian isoforms of the p85 adaptor subunit α and β (Otsu *et al.*, 1991; Escobedo *et al.*, 1991) and a p55γ adaptor subunit (Pons *et al.*, 1995). In addition, alternatively spliced variant (p55α and p50α) of p85α have been identified (Fruman *et al.*, 1996; Antonetti *et al.*, 1996; Inukai *et al.*, 1997). The 85kDa proteins contain two Src homology (nSH2 and cSH2) regions, which are linked by an inter-SH2 (iSH2) domain, a Src homology 3 (SH3) domain and a break point cluster region (BCR) (Table 1.2). The p55 subunit is highly homologous to the C-terminal half of the p85

subunits, but lacks the SH3 and BCR homology regions. SH2 domains mediate protein-protein interactions by binding phospho-tyrosine residues within a pYXXM motif. Amino acid residues 479-513 in the inter-SH2 domain have been shown to be the region of p85 which interacts with the p110 subunit (Klippel *et al.*, 1993; Dhand *et al.*, 1994a; Dhand *et al.*, 1994b). The roles of the SH3 and BCR domains are not well defined. SH3 domains bind proline rich regions containing the motif PXXP and the SH3 domain of p85 has been shown to bind to the adaptor protein Shc (Harrisonfindik *et al.*, 1995), the tyrosine kinase Lyn (Pleiman *et al.*, 1994) and the oncogene cbl (Soltoff and Cantley, 1996).

1.6 The Activation of Class I_A PI3K

Class I_A PI3Ks are activated in response to a number of cytokines and growth factors including IL-3, IL-4, SCF, GM-CSF and IL-5 (Gold *et al.*, 1994), insulin (Ruderman *et al.*, 1990), PDGF (Kaplan *et al.*, 1987), EGF, bFGF (Raffioni and Bradshaw, 1992). The p85 subunit is believed to regulate PI3K activity in two ways. Binding of p85 to p110 through the interaction of its iSH2 domain stabilises p110, protecting it from degradation, but also inhibits its kinase activity (Yu *et al.*, 1998). The p85 SH2 domains have a high selectivity for binding phosphorylated YXXM sequences that are present on some activated growth factor receptors. This targets PI3K to the membrane where its substrates are located. Phospho-tyrosine binding of the N-SH2 domain to the YXXM motif induces a conformational change that results in p110 catalytic activity (Yu *et al.*, 1998). For example, in the case of PDGF, ligand binding to the receptor results in autophosphorylation of the receptor on tyrosine residues in the YXXM motif in the interkinase domain. PI3K is activated by the interaction of p85 SH2 domains with these phosphotyrosine residues, which targets PI3K to its substrates in the membrane and causes a conformational change to the p110 subunit, which activates its catalytic activity (Coughlin *et al.*, 1989; Kazlauskas and Cooper, 1990; Escobedo *et al.*, 1991).

1.6.1 SCF-induced activation of PI3K

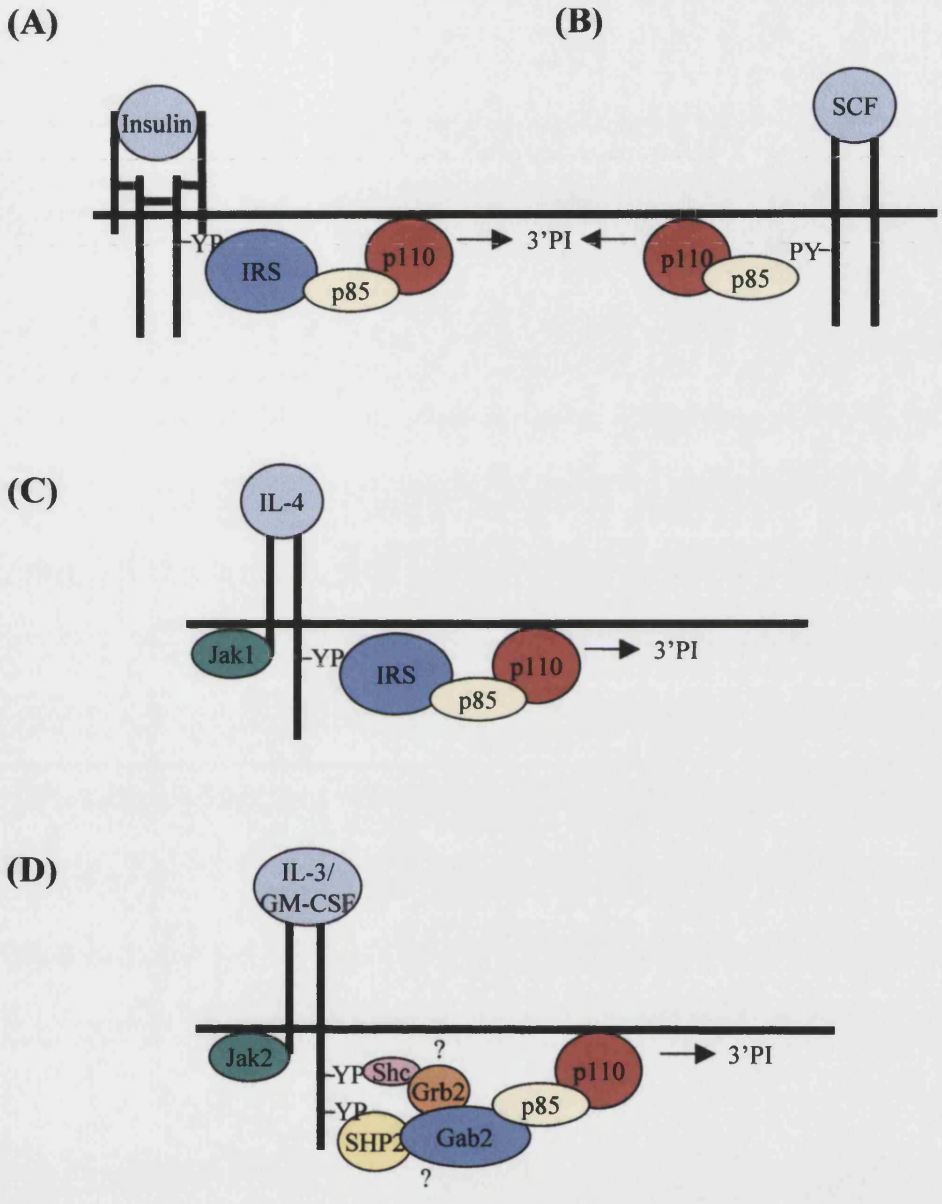
SCF activates PI3K by a similar mechanism to that of PDGF (Figure 1.10B). SCF binds to *c-kit*, which activates its tyrosine kinase domain resulting in the autophosphorylation of tyrosine residues. The kinase activity of c-kit is required for the activation of PI3K. In cells derived from mice homologous for the *W*42 mutant allele, in which *c-kit* does not possess intrinsic tyrosine kinase activity and therefore is not tyrosine phosphorylated, PI3K activity does not associate with *c-kit* upon SCF stimulation (Rottapel *et al.*, 1991). However, when a constitutively phosphorylated *c-kit* is expressed in cells, PI3K activity is bound in the absence of SCF stimulation (Rottapel *et al.*, 1991). The interaction of PI3K with *c-kit* is mediated through p85 association with phospho-Tyr^{719} in the motif pYMDM in the interkinase domain (Serve *et al.*, 1994). This is thought to activate PI3K by inducing the conformational change in p110 and by targeting PI3K to the membrane where it can interact with its lipid substrates. Mutation of *c-kit* residue Tyr^{719} to phenylalanine in mast cells results in the inhibition of SCF stimulated PI3K activity, as well as a partial inhibition of SCF induced proliferation and survival (Serve *et al.*, 1995). This implicates a role for PI3K in SCF supported growth and survival.

Other growth factor receptors do not contain YXXM docking sites for p85 SH2 domains. Instead they use adaptor proteins to target p85 to the receptor complex thus activating PI3K.

1.6.2 Insulin-induced activation of PI3K

Insulin and insulin-like growth factor-1 (IGF-1) activate PI3K by recruiting it to the receptor via the insulin receptor substrate (IRS) proteins (Figure 1.10A). IRS1 (Sun *et al.*, 1991) and IRS2 (also known as 4PS (Sun *et al.*, 1995) associate with the insulin and IGF-1 receptors through the interaction of their protein tyrosine binding (PTB) domain with an NPEY motif in the juxtamembrane region of the receptor (Wolf *et al.*, 1995). This results in the phosphorylation of the IRS proteins within YXXM motifs, which form sites for p85 SH2 domain association. This results in the translocation of PI3K to the membrane and the activation of the p110 catalytic subunit (Backer *et al.*, 1992).

Figure 1.10
Cytokine Activation of PI3K



The mechanism of activation of PI3K by **(A)** Insulin, **(B)** SCF, **(C)** IL-4 and **(D)** IL-3/GM-CSF are shown. Ligand binding to their receptors on the cell surface results in the tyrosine phosphorylation of the receptors and the recruitment of the p85 subunit of PI3K to the activated receptor complex either directly or through adapter proteins. See Section 1.18 details.

1.6.3 IL-4-induced activation of PI3K

Like insulin, IL-4 utilises the IRS proteins to target p85 via its SH2 domains and thus activate PI3K Figure 1.10C). However, unlike insulin, whose receptor contains intrinsic tyrosine kinase activity, the IL-4 receptor has no tyrosine kinase activity. IL-4 stimulation results in the activation of the kinases Jak1 and Jak3 (Witthuhn *et al.*, 1994; Welham *et al.*, 1995). In T-lymphocytes, IL-4 stimulation results in a complex formation between Jak1, IL-4R and IRS2 leading to the phosphorylation of all these proteins (Yin *et al.*, 1994). Studies in the haemopoietic cell line FDCP-2 found PI3K activity associated with the phosphorylated 170kDa IRS2 protein after IL-4, insulin and IGF-1 stimulation (Wang *et al.*, 1992) and p85 has also been shown to co-precipitate with IRS1 and IRS 2 after stimulation with IL-4 (Welham *et al.*, 1997; Jackson *et al.*, 1998). Collectively, these data suggest that PI3K might be sequestered to the membrane through the formation of a complex between p85, IRS1/2 and the IL-4R.

In common with the receptors for insulin and IGF-1, IL-4R contains an I4R motif (PLX_4NPXYXSXSD) in the juxtamembrane region that has been implicated in IRS binding to these receptors. Indeed, transfection of IRS-1 into 32D cells (which express neither IRS-1 or IRS-2) enables these cells to proliferate in response to a combination of IL-4 and insulin. However mutation of Y497 in the I4R domain of the IL-4 receptor to phenylalanine blocked the IL-4 induced IRS1 phosphorylation and proliferation of this cell line (Keegan *et al.*, 1994). Taken together these data point to a mechanism of IL-4 activation of PI3K whereby IL-4 binding to the IL-4R results in activation of Jak1 and its subsequent phosphorylation as well as receptor phosphorylation on a number of tyrosine residues including Y497 in the I4R domain. This creates a receptor-binding site for IRS2/IRS1 and results in phosphorylation in the YXXM motif that is recognised by the p85 adaptor subunit SH2 domains. This recruits PI3K to the membrane and results in the activation of the p110 catalytic PI3K subunit.

1.6.4 IL-3- and GM-CSF-induced activation of PI3K

βc is a common signalling component of the receptor complexes of both IL-3 and GM-CSF and for this reason the activation of PI3K by these cytokines will be discussed together (Figure 1.10D). IL-3 (Silvennoinen *et al.*, 1993) and GM-CSF (Quelle *et al.*, 1994) binding to their receptor complexes results in the tyrosine phosphorylation and activation of Jak2 and to a lesser extent Jak1, and this results in the tyrosine phosphorylation of a number of proteins including β_c. Two of these sites, Tyr^{577} and Tyr^{612} are thought to be important for the activation of PI3K as mutation of both these sites inhibited the IL-3 and GM-CSF-induced activation the downstream target of PI3K, PKB (Dijkers *et al.*, 1999). However, like the insulin and IL-4 receptors, β_c does not possess the YXXM motif required for p85 binding and it instead uses adaptor molecules to recruit p85 (Fantl *et al.*, 1992). Over recent years, with the cloning of the adaptor protein Gab2 (Gu *et al.*, 1998), much progress has been made in understanding the mechanisms by which IL-3 and GM-CSF activate PI3K. In response to IL-3 stimulation, Gab2 is heavily phosphorylated and binds to p85 (Shaw *et al.*, 1997; Gu *et al.*, 1998). It has also been shown to associate constitutively with Grb2, and after cellular stimulation with IL-3, to associate with the adaptor protein Shc and the phosphatase SHP2 (Shaw *et al.*, 1997; Gu *et al.*, 1998). Shc binds to βc through interactions with phosphorylated Tyr^{577} and to a lesser extent Tyr^{612} (Bone and Welham, 2000), while SHP2 binds to phosphorylated Tyr^{612} (Bone *et al.*, 1997) and these sites have been shown to be important for the phosphorylation of Gab2 in response to GM-CSF (Gu *et al.*, 2000). These data point towards two possible mechanisms by which the β_c receptor can recruit p85 to the membrane: through Shc association with phosphorylated Tyr^{577} and/or Tyr^{612}, or through SHP2 association with phosphorylated Tyr^{612}. There is some evidence to suggest that Grb2 is required for the interaction of Shc with p85 (Gu *et al.*, 2000). In NIH 3T3 cells expressing the hGM-CSFR cells, co-transfection of wild type Gab2 with wild type Grb2 resulted in the phosphorylation of Gab2. However, when Gab2 was co-transfected with Grb2 variants, which are defective in their SH2 or SH3 protein binding domains, Gab2 was not phosphorylated. This suggests that Grb2 may be required for the interaction of Gab2 and therefore p85 with β_c.

1.7 The Functions of PI3K

The study of PI3Ks and their role within the cell have been aided considerably by two unrelated specific pharmacological inhibitors wortmannin and LY294002. Wortmannin is a fungal metabolite that binds irreversibly to the p110 catalytic subunit (Ui *et al.*, 1995). LY294002 was discovered as an inhibitor of PI3K in a screen of quercetin (a naturally occurring bioflavaniod, which also inhibits PI3K activity) analogues. LY294002 inhibits PI3K by competing with ATP for binding to the active site and its effects are reversible (Vlahos *et al.*, 1994). These inhibitors have allowed the physiological responses, which are regulated by PI3Ks to be investigated. However, these compounds are active against most PI3Ks and therefore do not distinguish the functions of the different PI3K classes. In recent years a number of class I_A subunit variants have been constructed and these have provided insights into the functions of the class I_A PI3Ks. Of particular interest is the dominant negative Δp85 mutant. Δp85 does not bind the p110 catalytic subunit, but competes with endogenous p85 for binding to either receptor or adaptor YXXM motifs. Thus, when an excess of Δp85 is expressed the endogenous p85/p110 complex is not recruited to the membrane and activated.

Using these approaches, PI3Ks have been implicated in the regulation of many physiological processes. These include proliferation and mitogenesis (Brennan *et al.*, 1997; Craddock *et al.*, 1999; Hinton and Welham, 1999), cell survival (Yao and Cooper, 1995; Scheid *et al.*, 1995; Minshall *et al.*, 1996; Datta *et al.*, 1997; Dudek *et al.*, 1997; Khwaja *et al.*, 1997; Kennedy *et al.*, 1997; Songyang *et al.*, 1997; Scheid and Duronio, 1998), cytoskeletal reorganisation (Janmey, 1995; Hartwig, 1992), cell cycle regulation (Brennan *et al.*, 1997; Weinkove *et al.*, 1999), membrane trafficking (Brown *et al.*, 1995; Davidson, 1995) and membrane ruffling (Wennstrom *et al.*, 1994).

Recently, gene knockouts of a number of class I_A PI3K subunits have been made and these have given valuable insights into the functions of this class of PI3K. Gene targeted deletion of the first exon of p85α, which still allows the expression of p50α and p55α, gave rise to mice that were viable but had a B cell immunodeficiency. No impact was observed on T cells, which may be due to an increased expression of p50α in these cells (Suzuki *et al.*, 1999). However, p85α-p55α-p50α -/- mice die within days of birth (Fruman *et al.*, 1996). The survival and proliferation of chimeric B cells, which were in effect p85α-p55α-p50α -/- was also affected (Fruman *et al.*, 1996). Disruption of the p110α catalytic subunit resulted in embryonic lethality approximately halfway through term due to a severe defect in the proliferative capacity of the embryo (Bi *et al.*,

1999). However, p85α expression was upregulated in these embryos. This is thought to have a dominant negative effect on PI3K activity and may contribute to the lethal phenotype observed. Similarly, the expression of the p110α subunit was also affected in lymphocytes lacking p85α-p55α-p50α (Fruman *et al.*, 1996). Thus it appears that the knock out of any PI3K class I_A subunit will affect the expression of the other subunits and this should be remembered when interpreting the results of these studies.

1.8 Downstream Effectors of PI3K

The activation of PI3K leads to the production of 3'phosphoinositides, and these phospholipids act as second messengers and modulate the activity and/or location of downstream effectors of PI3K. Many of these proteins contain Pleckstrin homology (PH) domains, which facilitate their interaction with phospholipids. These include the Ser/Thr kinases PKB and PDK-1 (which will be discussed in detail later, Section 1.10 and 1.11.1), the Tec family of Tyr kinases, PLCγ2, PKC isoforms and the nucleotide exchange factor Vav.

The Tec proteins are a family of tyrosine kinases related to the src family. Unlike the src kinases they are not targeted to the membrane by an N-terminal myristylation sequence. However, they contain an N-terminal PH domain, which seems to substitute for N-myristylation to target Tec kinases to the membrane. Two members of this family are Brutons tyrosine kinase (BTK) and inducible T-cell kinase (ITK). The PH domains of both have been reported to bind to 3'phosphoinositides (August *et al.*, 1997; Bottomley *et al.*, 1998). Btk is critical for B-cell development and function and interestingly B-cells from Btk null mice have a similar phenotype to p85α knockouts (Khan *et al.*, 1995; Kerner *et al.*, 1995; Suzuki *et al.*, 1999; Fruman *et al.*, 1996).

PLCγ2 activity is also a regulated by phosphoinositides. The PLCγ2 PH domain is specific for $PI(3,4,5)P_3$. PLCγ2 has been reported to be a target of Btk (Scharenberg *et al.*, 1998) and it has been proposed that $PI(3,4,5)P_3$ may promote the association of PLCγ2 with Btk through the interactions the PH domain of each protein with $PI(3,4,5)P_3$ (Falasca *et al.*, 1998).

The lipid substrates and products of PI3K have been implicated in the regulation of some PKC family members. PKC isoforms, which have been implicated in PI3K signalling pathways, include δ (Le Good *et al.*, 1998), ε (Moriya *et al.*, 1996), η (Le Good *et al.*, 1998) and ζ (Standaert *et al.*, 1997; Le Good *et al.*, 1998).

The lipid products of PI3K have also been implicated in the regulation of GTP/GDP exchange factors (GEF) and GTPase-activating proteins (GAPS). For example, Vav, a GEF for Rac that stimulates the exchange of bound GDP for GTP, bound to and was directly controlled by substrates and products of phosphoinositide (PI) (Han *et al.*, 1998). This may explain why Rac acts downstream of PI3K in pathways leading to chemotaxis and membrane ruffling (Wennstrom *et al.*, 1994; Hawkins *et al.*, 1995).

1.9 Lipid Phosphatases

PI3K phosphorylates the 3'position of the inositol ring of phosphoinositides and these phospholipids act as second messengers that regulate a number of signalling pathways, some of which are discussed above. Unlike the hydrolysis of $PI(4,5)P_2$ by phospholipase-C to IP_3 and diacylglycerol (Kim *et al.*, 2000), the lipid products of PI3Ks are not substrates for phospholipases, instead their 3 or 5 phosphate groups are removed by phosphatases. Two phosphatases, PTEN and SHIP, have been identified which catalyse the removal of phosphate groups from PI3K lipid products.

1.9.1 PTEN

PTEN (phosphatase and tensin homologue deleted from chromosome 10, also known as MMAC1 and TEP1) was first identified in a search for new dual-specificity phospatases (Li *et al.*, 1997) and as a tumour suppressor gene (Li *et al.*, 1997; Steck *et al.*, 1997). It is one of the most common targets of mutation in human cancer (reviewed in Cantley and Neel, 1999). For example, germ-line mutations in PTEN cause the rare autosomal dominant inherited cancer syndromes Cowden disease, Lhermitte-Duclos disease and Bannayan-Zonana syndrome. These diseases are characterised by benign tumours in which differentiation is normal but cells are highly disorganised.

Sequence analysis of PTEN revealed a 47kDa dual-specificity phosphatase. However, PTEN was found to possess only weak tyrosine and serine/threonine phosphatase activity (Li and Sun, 1997; Myers *et al.*, 1997). Studies by Maehama and Dixon showed that PTEN could de-phosphorylate $PI(3,4,5)P_3$ and to a lesser extent other phosphoinositols *in vitro* (Maehama and Dixon, 1998). They showed that overexpression of PTEN decreased the levels of $PI(3,4,5)P_3$ produced following insulin stimulation without affecting the activity of PI3K, and overexpression of phosphatase-inactive PTEN led to the accumulation of $PI(3,4,5)P_3$.

Embryonic fibroblasts from PTEN -/- mice exhibit a marked decrease in sensitivity to apoptosis induced by a variety of stimuli including UV, heat shock and TNFα

(Stambolic *et al.*, 1998). This sensitivity is restored by introduction of wtPTEN. PTEN -/- cells also had increased PKB activity that was reduced by re-introduction of PTEN. Similarly, an active mutant of PKB suppressed apoptosis induced by PTEN over-expression.

PTEN over-expression also results in a decrease in cell proliferation and the accumulation of cells in G1 with decreased progression into S phase (Li and Sun, 1997). This was accompanied by an increase in the protein level of the cyclin-CDK inhibitor p27/Kip1 and followed the PTEN mediated inhibition of PKB activity. Co-expression of a constitutively active form of PKB in a renal carcinoma cell line was sufficient to over-ride a PTEN induced G1 block (Ramaswamy *et al.*, 1999). These results suggest that one function of PTEN is to negatively regulate the PI3K pathways leading to both proliferation and survival.

1.9.2 SHIP

SHIP-1 (SH2-containing Inositol 5'-Phosphatase-1) was first identified as a tyrosine-phosphorylated protein observed following stimulation of haemopoietic cells by various cytokine and growth factors (Rohrschneider *et al.*, 2000). A number of SHIP splice variants have been identified and they encode proteins of between 108 and 145kDa. SHIP catalyses the removal of the phosphate group from the 5' position of both $PI(3,4,5)P_3$ and $PI(1,3,4,5)P_4$ (Damen *et al.*, 1996; Lioubin *et al.*, 1996). It is believed that the activity of SHIP is regulated through its location in the cell and that upon growth factor stimulation cytoplasmic SHIP is targeted to the plasma membrane near its lipid substrates.

The 3' position of the inositol phospholipid must be phosphorylated before SHIP can dephosphorylate the 5' position, and this has lead to the idea that SHIP may act sequentially with PI3K (Damen *et al.*, 1996). Studies of SHIP-1 knock out mice found an increase in myeloid cell proliferation that was associated with increased $PI(3,4,5)P_3$ production and wortmannin sensitive activation of PKB (Liu *et al.*, 1999). Neutrophils and bone marrow-derived mast cells from SHIP-1 null mice were also more sensitive to apoptosis (Liu *et al.*, 1999). Therefore, SHIP appears to play a negative role in myeloid proliferation perhaps through the down regulation of the PI3K/PKB pathway.

1.10 Protein Kinase B

Over recent years, PKB has been the focus of intense research. The reasons for this are manifold; PKB is a major target of the PI3K signalling pathway and has been

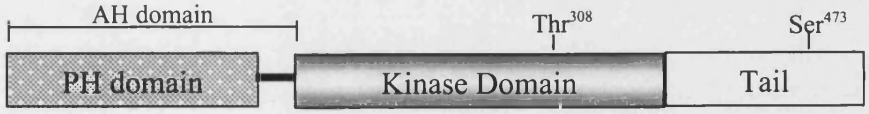
implicated in the regulation of many PI3K-mediated events including proliferation, survival, and several metabolic actions of insulin. However, the area, where perhaps most interest has focussed, is in characterising the role of PKB in cellular survival.

Protein kinase B (PKB, also known as Akt and Rac), was isolated by three independent groups in 1991. Two of these (Coffer and Woodgett, 1991; Jones *et al.*, 1991) identified PKB from its homology to PKA and PKC hence the names were coined PKB and RAC (related to A and C). The third group (Bellacosa *et al.*, 1991) isolated the cellular homologue of *v-Akt,* an oncogene from the AKT8 acutely transforming retrovirus isolated from a rodent T-cell lymphoma (Staal, 1987). PKB is a serine/threonine kinase, which has 68% and 73% homology to PKA and PKC respectively. Three isoforms have been identified PKBα (Jones *et al.*, 1991; Coffer and Woodgett, 1991; Bellacosa *et al.*, 1991), PKBβ (Cheng *et al.*, 1992) and PKBγ (Konishi *et al.*, 1995; Nakatani *et al.*, 1999b; Brodbeck *et al.*, 1999) which are 60 kDa proteins although a C-terminal truncated 55kDa PKBγ isoform was isolated from rat cells (Konishi *et al.*, 1995). All three isoforms are ubiquitously expressed in mammals, although the levels of expression vary among tissues. PKBα is the predominant isoform in most tissues, the highest levels of PKBβ expression is in skeletal muscle, heart, liver and kidney (which suggests a role in insulin signalling), and PKBγ expression levels are highest in testis and brain (Jones *et al.*, 1991; Bellacosa *et al.*, 1993; Altomare *et al.*, 1995; Konishi *et al.*, 1995; Brodbeck *et al.*, 1999; Nakatani *et al.*, 1999a).

There is approximately 80% homology between the three isoforms and they share the same structural features (Figure 1.11). They contain an N-terminal pleckstrin homolgy (PH) domain (residues 1-106). PH domains bind the charged headgroups of phosphatidylinositols and are involved in mediating interactions with phospholipids. The PH domain is within a region often referred to as the AH (Akt homology) region which spans residues 1-148. The catalytic Ser/Thr kinase domain (residues 148-412) lies between the AH domain and the C-terminal tail region. This domain belongs to the AGC subfamily of protein kinase domains (where AGC is short for the cyclic AMP-dependent protein kinase, cyclic GMP-dependent protein kinase and protein kinase C). The C-terminal of PKB consists of a hydrophobic tail region, which is thought to be involved in the regulation of PKB activity.

Figure 1.11

Schematic representation of PKB



The overall structure of PKB is shown. The Pleckstrin homology (PH) domain, which is involved in phospholipid binding, the Akt homology region (AH domain), the kinase domain and the C-terminal tail regions are indicated. Phosphorylation at Thr^{308} (Thr^{309} in PKBβ, Thr^{305} in PKBγ) and Ser^{473} (Ser^{474} in PKBβ and Ser^{472} in PKBγ) are needed for maximal activation.

1.11 Activation of PKB

PKB is activated in response to numerous stimuli including growth factors, phosphatase inhibitors, cAMP agonists and stress conditions (Reviewed in Coffer *et al.*, 1998; Datta *et al.*, 1999; Vanhaesebroeck and Alessi 2000). Growth factors that activate PKB include PDGF (Franke *et al.*, 1995), insulin (Cross *et al.*, 1995), EGF, bFGF (Burgering and Coffer, 1995), IL-3, (Franke *et al.*, 1995; Burgering and Coffer, 1995; Songyang *et al.*, 1997; del Peso *et al.*, 1997; Datta *et al.*, 1997; Scheid and Duronio, 1998; Hinton and Welham, 1999; Craddock *et al.*, 1999), SCF (Scheid and Duronio, 1998; Blume-Jensen *et al.*, 1998; Hinton and Welham, 1999), IL-4, and GM-CSF (Scheid and Duronio, 1998; Hinton and Welham, 1999). The activation of PKB is inhibited by the PI3K inhibitors wortmannin and LY294002 as well as Δp85 (a dominant negative PI3K mutant) which indicates that the growth factor-induced activation of PKB is mediated through PI3K (Kauffman-Zeh *et al.*, 1997; Kennedy *et al.*, 1997; Songyang *et al.*, 1997; Khwaja *et al.*, 1997; Craddock *et al.*, 1999; Hinton and Welham, 1999).

Both phosphorylation and the PH domain appear to be important for the growth factor-induced activation of PKB. PKB is phosphorylated on 4 sites: Ser^{124}, Thr^{308}, Thr^{450} and Ser^{473} (Alessi *et al.*, 1996a). Ser^{124} and Thr^{450} are phosphorylated under basal conditions while phosphorylation of Thr^{308} and Ser^{473} is induced by growth factor stimulation. Thr^{308} (Thr^{309} in PKBβ and Thr^{305} in PKBγ) is located in the activation loop of the kinase

domain and Ser^{473} (Ser^{474} in PKBβ and PKBγ) is in the C-terminal tail region. Phosphorylation of both these residues is required for optimal activation of PKB (Alessi *et al.*, 1996a). Mutation of either Ser^{473} or Thr^{308} to alanine reduces the growth factor induced PKB activation, while mimicking the negative charge of the phosphate group, by mutation to aspartate, results in PKB activation in unstimulated cells (Alessi *et al.*, 1996a). In recent years much work has been done to identify the kinase(s) responsible for the phosphorylation of Ser^{473} and Thr^{308}, and some candidates have been identified.

1.11.1 PDK-1

A kinase which phosphorylates PKB on Thr^{308} was identified and purified and named PI(3,4,5)P_3 dependent kinase-1 (PDK-1) because of its apparent dependence on phosphoinositides (Alessi *et al.*, 1997; Stokoe *et al.*, 1997). PDK-1 is a ubiquitously expressed 63kDa protein consisting of a PH domain and a kinase domain belonging to the AGC protein kinase subfamily. While PDK-1 is dependent on the lipid products of PI3K to phosphorylate PKB (Alessi *et al.*, 1997), PDK-1 itself appears to be constitutively active (Alessi *et al.*, 1997; Pullen *et al.*, 1998; Casamayor *et al.*, 1999). PDK1 activity is dependent on its phosphorylation at Ser^{241}. However, IGF-1 does not increase the phosphorylation of PDK-1 at Ser^{241} or indeed any site (Casamayor *et al.*, 1999). This suggests that PDK1 is constitutively active, which is surprising given that PKB and other substrates of PDK-1 are not phosphorylated in the absence of growth factor.

On its own PDK-1 is incapable of phosphorylating PKB at Ser^{473} but there is evidence to suggest that under some conditions it becomes a Ser^{473} kinase. Balandran *et al.* (2000) reported that PDK-1 can interact with a C-terminal fragment of PRK-2 (protein kinase C related kinase-2), and this interaction converts PDK-1 to a kinase that is capable of phosphorylating PKB at Ser^{473}. This interaction also converts PDK-1 into a form that is activated by the presence of the PI3K products PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. However, in PDK-1 -/- ES cells PKB was constitutively phosphorylated at Ser^{473}, which suggests that other kinases must exist that phosphorylates this site *in vivo* (Williams *et al.*, 2000).

1.11.2 ILK (Integrin-Linked Kinase)

ILK is a serine/threonine kinase that was first identified by its binding to the cytoplasmic domain of the β1, β2, and β3 integrin subunits and is involved in the integrin signalling pathway (Altomare *et al.*, 1995). Over-expression of ILK in

epithelial cells results in anchorage-independent cell survival and cell cycle progression (Radeva *et al.*, 1997) and tumorigenesis in nude mice (Wu *et al.*, 1998). ILK has also been implicated as a regulator of the Wnt-signalling pathway, which is involved in cell fate determination (Novak *et al.*, 1997).

The intergrin linked kinase (ILK) has also been reported to phosphorylate PKB on Ser^{473} (Delcommenne *et al.*, 1998). *In vitro* kinase assays showed that ILK can directly phophorylate PKB on Ser^{473} and cotransfection of PKB and a kinase deficient ILK into 293 cells inhibited Ser^{473} phosphorylation. Wortmannin and LY294002 inhibited the insulin and cell-fibronectin-induced activation of ILK in epithelial cells indicating that the activation of ILK by these stimuli is mediated by PI3K. However, Lynch *et al.* (1999) have reported that ILK regulates the phosphorylation of Ser^{473} through an indirect mechanism, perhaps by acting as an adaptor. Taken together these data suggest that ILK may be involved in the insulin- and integrin-induced phosphorylation of PKB and it will be interesting to see if it plays a similar role in the activation of PKB by cytokines.

1.11.3 PH Domain

The PH domain of PKB has been shown to be critical to the activation of PKB (Franke *et al.*, 1995; Franke *et al.*, 1997; Sable *et al.*, 1998). PKB binds to the phospholipid products of PI3K through an interaction with the PH domain of PKB (James *et al.*, 1996; Frech *et al.*, 1997; Franke *et al.*, 1997), which results in the translocation of PKB from the cytosol to the inner surface of the plasma membrane (Andjelkovic *et al.*, 1997; Meier *et al.*, 1997; Wijkander *et al.*, 1997; Sable *et al.*, 1998; Currie *et al.*, 1999). Oncogenic *v-Akt*, which is constitutively active, contains an N-terminal membrane targeting gag fusion derived from a retroviral gag protein (Bellacosa *et al.*, 1991). Similarly, targeting PKB to the cellular membrane either by the fusion of a gag or src myristylation sequence results in the constitutive activation of PKB through increased phosphorylation (Franke *et al.*, 1995; Andjelkovic *et al.*, 1997; Kohn *et al.*, 1996a). These data suggest that translocation to the plasma membrane is important for the activation of PKB, perhaps because it targets PKB to its activating kinases. In resting cells PDK-1 is mostly cytosolic (Anderson *et al.*, 1998; Currie *et al.*, 1999) and is constitutively active (Alessi *et al.*, 1997; Pullen *et al.*, 1998; Casamayor *et al.*, 1999). However, it is dependent on the presence of $PI(3,4,5)P_3$ or $PI(3,4)P_2$ to catalyse the phosphorylation of Thr^{308} on PKB (Alessi *et al.*, 1997). Upon cellular stimulation, PDK-1 has been reported to translocate to the plasma membrane and the

co-localisation of PKB and PDK-1 may allow for the activation of PKB (Anderson *et al.*, 1998).

The PH domain of PKB is also believed to regulate the activity of PKB by controlling the access of kinase(s) to the phosphorylation sites Ser^{473} and/or Thr^{308}. In the absence of $PI(3,4,5)P_3$ PKB is not phosphorylated by PDK-1 (Alessi *et al.*, 1997). Mutants with an R25C mutation, that prevents the PH domain from binding phospholipids, are not activated insulin (Sable *et al.*, 1998). However, when the PH domain is deleted (ΔPH-PKB) this PKB variant is constitutively active due to increased phosphorylation (Kohn *et al.*, 1996a; Stokoe *et al.*, 1997; Sable *et al.*, 1998). These findings are consistent with the hypothesis that phospholipid binding of the PH domain results in a conformational change in PKB that allows the activating kinase(s) access to Ser^{473} and/or Thr^{308}. The deletion of the PH domain is thought to release the conformational restraints on PKB allowing the activating kinase(s) access to Ser^{473} and Thr^{308}.

The PH domain has also been reported to facilitate the formation of PKB homo-multimers (Datta *et al.*, 1995; Franke *et al.*, 1997). PKB has been reported to interact with TCL1 (T-cell leukaemia/lymphoma 1), the product of the oncogene *Tcl1*, which is involved in the development of T-cell leukaemia (Pekarsky *et al.*, 2000; Laine *et al.*, 2000). The interaction of PKB with TCL1 increases PKB activity and translocation to the nucleus, which leads to the suggestion that it acts as a coactivator, enhancing PKB activity (Pekarsky *et al.*, 2000; Laine *et al.*, 2000). *In vivo*, TCL1 forms trimers, which associate with PKB and thus facilitate the oligermerisation of PKB (Laine *et al.*, 2000).

1.12 Funtions of PKB

Many potential substrates of PKB have now been identified, and these have implicated PKB in the regulation of a number of physiological processes including cell-cycle regulation (Medema *et al.*, 2000), survival (Dudek *et al.*, 1997; Kennedy *et al.*, 1997; Songyang *et al.*, 1997; Datta *et* al., 1997; Sabbatini and McCormick, 1999; Tang *et al.*, 2000), annoikis (Khwaja *et al.*, 1997), proliferation (Brennan *et al.*, 1997), glycogen synthesis (Cross *et al.*, 1994), glucose transport (Kohn *et al.*, 1996b; Tanti *et al.*, 1997; Hajduch *et al.*, 1998) glycolysis (Deprez *et al.*, 1997) and protein synthesis (Ueki *et al.*, 1998; Gingras *et al.*, 1998).

Much interest and research has focussed on the effects of PKB on cell survival. PKB was first shown to be involved in the regulation of apoptosis when it was found that the expression of a dominant negative PKB inhibited the IGF-1-mediated survival of cultured cerebellar granule cells (Dudek *et al.*, 1997). Conversely, the expression of wild type PKB or a constitutively active PKB variant negated the dependence of these cells on IGF-1 for survival. Since then, PKB has been implicated in the prevention of apoptosis induced by growth factor withdrawal (Dudek *et al.*, 1997; Kennedy *et al.*, 1997; KauffmanZeh *et al.*, 1997; Songyang *et al.*, 1997; Datta *et al.*, 1997), uv irradiation and DNA damage (Kulik and Weber, 1998), matrix detachment (Khwaja *et al.*, 1997), fas ligand (Rohn *et al.*, 1998) and TGFβ (Chen *et al.*, 1998).

In the absence of a growth signal (ie cytokine) haemopoietic cells cease to proliferate and undergo apoptosis. In the mid nineties Scheid and coworkers had demonstrated that the stimulation of the mast cell line MC9, with various cytokines resulted in the activation of PI3K (Gold *et al.*, 1994). Using the PI3K inhibitors, wortmannin and LY294002, this group then demonstrated that PI3K was required for the survival of MC9 cells mediated by IL-3, IL-4 and SCF (Scheid *et al.*, 1995). With the discovery that PKB is activated by PDGF through a PI3K-mediated pathway (Franke *et al.*, 1995), groups began to investigate PKB as a potential downstream regulator of the cytokine-induced and PI3K mediated survival of haemopoietic cells.

Two groups demonstrated that the expression of a constitutively active PKB variant was sufficient to delay the onset of apoptosis induced by cytokine withdrawal in the pre-B cell line BaF/3 (Ahmed *et al.*, 1997) and the myeloid progenitor cell line 32D (Songyang *et al.*, 1997). These results suggested that PKB might be involved in the PI3K-mediated survival signal, which is generated by cytokines, but the mechanisms of this were not known. Del Peso *et al.*, (1997), showed that in the lymphoid progenitor cells line FL5.12, the IL-3-induced phosphorylation of the pro-apoptotic Bcl-2 family

member, Bad, was blocked by the PI3K inhibitors, LY294002 and wortmannin. They, and others (Datta *et al.*, 1997), then identified Bad as a direct substrate of PKB. Collectively, these results suggested that the PI3K/PKB pathway is important to the cytokine-mediated survival of haemopoietic lineages.

The role that PKB plays in regulating the proliferation of cells is less well defined than the one it plays in apoptosis. However, there is some evidence to suggest that it may be involved in the proliferation of haemopoietic lineages. Ahmed *et al.*, (1997) showed that the expression of a constitutively active PKB variant in BaF/3 cells transfected with an IL-2 R mutant that is defective in PI3K signalling, increased the number of cells that became factor independent. While in T lymphocytes, the expression of an active PKB variant was shown to be sufficient to induce E2F activity (Brennan *et al.*, 1997). PI3K inhibition in these cells was shown to inhibit the phosphorylation of pRb, induction of cyclin D3, and degradation of $p27^{kip1}$. These results suggested that the PKB might also be involved in the proliferation of haemopoietic cells.

1.13 Downstream of PKB

Many potential substrates of PKB have now been identified. The evidence implicating these proteins as effectors of PKB is varied and may include the effects of dominant negative and positive PKB variants, the effects of PI3K inhibition and activation and the identification of the phosphorylation sites. Ideally, several lines of evidence should be considered when identifying substrates. The minimum sequence motif that is recognised and phosphorylated by PKB has been determined as RXRXXS/TH where X is any amino acid and H is a bulky hydrophobic residue (phenylalanine or leucine) (Alessi *et al.*, 1996b) and many of the substrates that have been identified contain motifs that conform to this consensus sequence. Table 1.3 lists the potential substrates of PKB identified to date and some of the key downstream targets of PKB and the implications of their activation or inactivation in relation to cell physiology are discussed below.

1.13.1 Glycogen Synthase Kinase-3

Glycogen synthase kinase-3 (GSK-3) was first identified as an enzyme that regulates glycogen synthesis in response to insulin stimulation. Subsequently, it has also been implicated in a number of biological processes including metabolism, gene expression, cell fate determination, proliferation and survival. GSK-3 is a ubiquitously expressed

protein of 51kDa (GSK-3α) and 47kDa (GSK-3β). On stimulation with growth factor, GSK-3 is rapidly phosphorylated resulting in the inhibition of GSK-3 kinase activity and the subsequent increase in glycogen synthesis (Cross *et al.*, 1994). PKB was shown to phosphorylate GSK3 on Ser^{21} (GSK-3α) or Ser^{9} (GSK-3β), and phosphorylation of this site results in the inhibition of GSK3 (Cross *et al.*, 1995; Shaw *et al.*, 1997).

Table 1.3
PKB Substrates

Protein Target	Function	Reference
GSK3α/β	Glycogen synthesis	Cross *et al.*, 1995
6-phosphofructose-2-kinase	Glycogen synthesis	Deprez *et al.*, 1997
Phosphodiesterase 3B	Inhibition of glycogen degradation	Kitamura *et al.*,1999
Forkhead	Survival/proliferation	Brunet *et al.*, 1999 Kops *et al.*,1999
BAD	Survival	Del peso *et al.*, 1997
Caspase-9	Survival	Cardone *et al.*, 1999
IKKα	Survival/proliferation	Ozes *et al.*, 1999 Romashkova *et al.*, 1999
Endothelial nitric oxide synthase	NO production	Fulton *et al.*,1999 Dimmeler *et al.*, 1999
Mammalian Target of Rapamycin	Proliferation	Scott *et al.*, 1998
IRS	Insulin signalling	Li *et al.*, 1999
BRAC1	DNA repair	Altiok *et al.*, 1999
Raf	Survival/proliferation	Zimmermann *et al.*, 1999

1.13.2 Bad

Another direct substrate of PKB identified, Bcl-2/Bcl-X_L-antagonist, causing cell death (Bad), is a member of the Bcl-2 family of proteins that are intracellular regulators of programmed cell death/apoptosis (refer to Section 1.3). Overexpression of the anti-apoptotic members (e.g. Bcl-2, Bcl-X_L) prevents cell death, while overexpression of the pro-apoptotic members (e.g. Bax, Bak, Bad) promotes cell death. Family members are able to form homo- and hetero-dimers through the interactions of their Bcl-homology 3 (BH3) domains. These interactions and the relative level of each protein in the cell, is thought to determine the fate of the cell. Bcl-2 family members are also regulated by survival factor-induced phosphorylation. Phosphorylation of Bcl-2 family members alters their binding properties thus providing the appropriate signal for survival or apoptosis.

Bad forms heterodimers with both Bcl-2 and Bcl-X_L, neutralising their anti-apoptotic affects and thus promoting cell death (Zha *et al.*, 1997, Ottalie *et al.*, 1997, Kelekar *et al.*, 1997). However, after stimulation with growth factor, BAD becomes phosphorylated on a number of residues including Ser^{112}, Ser^{136} (Zha *et al.*, 1996), Ser^{155} (Lizcano *et al.*, 2000; Zhou *et al.*, 2000) and Ser^{170} (V. Duronio, Canada, personal communication). The phosphorylation of Bad results in Bad associating with the protein adaptor 14-3-3 (Zha *et al.*, 1996). This prevents its interaction with Bcl-X_L allowing Bcl-X_L to promote survival of the cell. There has been great interest in identifying the kinases that phosphorylate each of these residues. Early reports indicated that Bad is phosphorylated on Ser^{136} by PKB in response to stimulation by IL-3 and PDGF (del Peso *et al.*, 1997; Datta *et al.*, 1997) but the kinase(s) responsible for phosphorylation at the other sites were unknown.

Recently, a number of papers have been published identifying at least some of the other kinases involved. Ser^{112} phosphorylation is dependent on the integrity of the MAPK pathway (Scheid *et al.*, 1999; Bonni *et al.*, 1999; Tan *et al.*, 1999; Fang *et al.*, 1999; Lizcano *et al.*, 2000). MAPKAP-K1 (mitogen-activated protein kinase activated protein kinase-1, also known as RSK), which is an immediate downstream target of Erk1 and 2, was found to phosphorylate Ser^{112} in co-transfection experiments in 293 cells (Bonni *et al.*, 1999; Tan *et al.*, 1999; Fang *et al.*, 1999). Bad is phosphorylated preferentially at Ser^{155} by PKA *in vitro* and is the only residue that becomes phosphorylated when cells are exposed to cAMP-elevating agents such as forskolin and

IBMX (Lizcano *et al.*, 2000; Tang *et al.*, 2000). In addition to this, phosphorylation of Bad at Ser^{155} was prevented by H89, an inhibitor of PKA (Zhou *et al.*, 2000).

1.12.3 Caspase-9

Caspases are the mammalian homologues of the *C. elegans* Ced-3 (refer to Section 1.3). They are activated from their inactive pro-forms to their active forms by a sequential cascade of cleavage by caspase family members. Caspase-3, which is an effector caspase, is activated by cleavage of procaspase-3 into its active form, caspase-3, by the initiator caspase-9 (Slee *et al.*, 1999). Association of caspase-9 with Apaf-1 (a ced-4 homologue) and cytochrome c binding protein results in its activation by an auto-cleavage mechanism (Li *et al.*, 1997; Zou *et al.*, 1997). One way that PKB may regulate the activity of caspase-9 is through Bcl-X_L. PKB phosphorylation of Bad prevents its association with Bcl-X_L (Zha *et al.*, 1996). This prevents the release of cytochrome c from the mitochondria (Kennedy *et al.*, 1999), which prevents the formation of the cytochrome c/Apaf-1/caspase-9 complex that results in the activation of caspase-9 (Li *et al.*, 1997). However, caspase-9 may also be a direct target of PKB (Cardone *et al.*, 1998). Caspase-9 is phosphorylated by PKB at Ser^{196} and extracts from cell lines over-expressing PKB block cytochrome c-mediated caspase-9 activation *in vitro*. It is not clear how phosphorylation of caspase-9 results in its inactivation, although it appears to inactivate the intrinsic catalytic activity of caspase-9. However, PKB phosphorylation sites are not found in murine, monkey and rat caspase-9 and activated PKB will not phosphorylate murine caspase-9 *in vitro* (Fujita *et al.*, 1999). This suggests that inhibition of caspase-9 activation by PKB-dependent phosphorylation may not be relevant to all species.

1.12.4 Forkhead

Studies in *Caenorhabditis elegans* showed that mutation in either the insulin/IGF receptor homologue *daf-2* or the PI3K homologue *age-1* cause animals to developmentally arrest in the dauer lavae stage, and to live longer. Mutations in the forkhead transcription factor homologue DAF-16, suppressed the developmentally arrested larval, or dauer, and the longevity phenotypes of both the *daf-2* and *age-1* mutants, indicating that DAF-16 is a negatively regulated downstream target of insulin-like signalling in *C. elegans*. Genetic screens of these mutants revealed that the PKB homologues AKT-1 and AKT-2 are required for signalling from both DAF-2 and AGE-1 (Paradis and Ruvkun, 1998). Inhibition of AKT-1 and AKT-2 resulted in constitutive

dauer formation but this effect was not seen in animals containing *daf-16* null mutations. This was significant because it identified the DAF-16 (forkhead) transcription factor as a major target of the PI3K/PKB pathway in *C. elegans*.

In mammalian systems three members of the forkhead family have been identified; FKHR, FKHRL1/AF6q21 and AFX. They share a forkhead domain of 100aa, which mediates their interaction with DNA and a C-terminal transcriptional activation domain (Kops and Burgering, 1999). There are three PKB phosphorylation consensus sites within the Forkhead proteins, Thr^{24}, Ser^{256} and Ser^{319} in FKHR (Tang *et al.*, 1999). PKB is able to phosphorylate all of them *in vitro* although Ser^{256} appears to be the major site (Rena *et al.*, 1999; Nakae *et al.*, 1999). The expression of a constitutively active PKB is sufficient to induce the phosphorylation of the Forkhead proteins at all three sites (Rena *et al.*, 1999). Phosphorylation of forkhead isoforms FKHR/ FKHRL1 at Ser^{256}/Ser^{253} and Thr^{32} is induced by insulin, IGF-1, EPO and inhibited by the PI3K inhibitor LY294002 (Guo *et al.*, 1999; Kashii *et al.*, 2000).

PKB is thought to mediate forkhead family member function by regulating its subcellular localization. Non-phosphorylated forkhead proteins are located in the nucleus but when phosphorylated they are found in the cytoplasm (Biggs *et al.*, 1999; Brunet *et al.*, 1999; Kops and Burgering, 1999). A mutant of FKHRL1, in which all three PKB phosphorylation sites are mutated to alanine, is located in the nucleus even in the presence of survival factors (Biggs *et al.*, 1999). This indicates that the subcellular localization of forkhead proteins is regulated by their phosphorylation by PKB. Phosphorylated FKHRL1 has been shown to interact with 14-3-3 proteins (Brunet *et al.*, 1999). In the absence of phosphorylation, forkhead family members in the nucleus bind to specific DNA elements. FKHRL1 binds to sites in the Fas ligand (FasL) promoter and induces expression of a reported gene driven by the FasL promoter (Brunet *et al.*, 1999). This points to a hypothesis whereby in the absence of survival factors, PKB is inactive allowing forkhead family members to induce the transcription of FasL that induces apoptosis.

There is also evidence to suggest that forkhead proteins mediate gene expression through Insulin Response Sequences (IRS). These sequences are contained within the promoter regions of a number of insulin-regulated genes including those that encode insulin-like growth factor binding protein-1 (IGFBP-1), apolipoprotein CIII, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase. Reporter gene studies using the IGFBP-1 promoter demonstrate that FKHR stimulates promoter activity through an IRS motif and that phosphorylation of Ser^{256} by PKB is necessary

and sufficient for insulin to disrupt IRS-dependent transactivation by FKHR (Guo *et al.* 1999). This suggests that the PKB/forkhead pathway may be important in regulating the transcriptional activity of genes containing IRS sequences.

A recent report has linked PKB regulation of forkhead proteins with proliferation. Expression of AFX blocks cell-cycle progression at G_1 by transcriptionally upregulating the cell-cycle inhibitor $p27^{kip1}$. Insulin stimulation resulted in activation of PKB, phosphorylation of FKHR-L1 and a reduction of $p27^{kip1}$ expression. Inhibition of PI3K and PKB with LY294002 caused a reduction in FHKR-L1 phosphorylation and an increase in $p27^{kip1}$ levels (Medema *et al.*, 2000).

1.12.5 IκB Kinase

PKB has been reported to phosphorylate IκB kinaseα (IKKα) on Thr^{23} resulting in its activation by both TNF and PDGF (Ozes *et al.*, 1999; Romashkova and Makarov, 1999). This pathway is inhibited by LY294002, wortmannin and the dominant negative PI3K Δp85 indicating that it is mediated through PI3K. The activation of IKK leads to the phosphorylation of IκB and targets it for degradation. The degradation of IκB results in the activation and translocation to the nucleus of the transcription factor NFκB. NFκB regulates the transcription of a number of factors including pro-inflammatory cytokines and the induction of c-myc (Baldwin, 1996).

1.13 Aims and Objectives

Over recent years PKB has been widely implicated as a positive regulator of cellular proliferation and survival in a number of systems (Reviewed in Vanhaesebroeck and Alessi 2000, Coffer *et al.*, 1998, Datta *et al.*, 1999). This has lead to the generally accepted view that PKB is both necessary and sufficient to drive the long-term growth and survival of cells. However, whether this is indeed the case in all systems had not been investigated. This project aimed to address this issue by investigating the role of PKB in the survival and proliferation of haemopoietic cells in response to specific cytokines. To achieve this broad objective, two lines of investigation were undertaken. The first compared the cytokine-induced activation of endogenous PKB to the growth and survival of haemopoietc cells, and the second focussed on the role of PKB in the IL-3-mediated growth and survival of BaF/3 cells by expressing PKB variants in these cells and to characterise their effects.

Using the first approach, I hoped to determine

- If PKB is activated in these haemopoietic cells in response to cytokine-stimulation
- If this PKB activation is mediated through PI3K
- Whether PKB activity correlated with the cytokine-induced growth and proliferation of these cells
- What downstream effectors of PKB are involved in PKB signalling in these cells

I also hoped to focus specifically on the role of PKB in the IL-3 dependent BaF/3 cell line. My objectives here were to

- Create BaF/3 cell lines which inducibly express PKB variants
- To use these variants to investigate the dependence of these cells on IL-3 for their continued proliferation and survival
- To investigate the PKB signalling pathways activated in these cells.

Chapter 2
Materials and Methods

2.1 Molecular Biology Techniques

2.1.1 Phenol/Chloroform extractions

The DNA solution was extracted in an equal volume of buffer-saturated phenol:chloroform (1:1) and centrifuged for 1 minute at full speed in a Heraeus microcentrifuge. The aqueous phase was then transferred to a clean tube and re-extracted with an equal volume of chloroform. After centrifugation for 1 minute, the aqueous phase was transferred to a clean tube, ready for ethanol precipitation.

2.1.2 Ethanol precipitation of DNA

2 volumes of 100% ethanol and 0.1 volume of sodium acetate (3M, pH 5.5) were added to the solution containing the DNA to be precipitated. The solution was mixed by inversion, cooled on ice for 1-5 minutes and the precipitated DNA pelleted in a Heraeus microcentrifuge at 4°C for 10 minutes at full speed. The ethanol was aspirated off and the pellet washed once in 70% ethanol. The pellet was left to air dry at room temperature before being resuspended in water and stored at -20°C or resuspended in electroporation buffer for transfection.

2.1.3 Preparation of competent *E. coli*

A frozen stock of XL-1BL (Stratagene) was streaked onto a luria broth agar plate (10g bacto-tryptone, 5g yeast extract, 5g NaCl, 15g bacto-agar), inverted and incubated overnight at 37°C. A single colony was then used to inoculate 5ml LB (10g bacto-tryptone, 5g yeast extract, 5g NaCl), and was incubated overnight at 37°C in a shaking incubator. The following day, the bacteria were subcultured 1:100 in 100ml LB broth and grown until an OD_{550} of 0.48 was reached. The culture was then chilled on ice for 5 minutes and centrifuged in Beckman M5 centrifuge with a JA-14 rotor at 5000rpm at 4°C for 10 minutes. The supernatant was removed and the bacteria resuspended in 0.4 volume (40 ml) of TfbI (30mM KCl, 100mM RbCl, 10mM $CaCl_2$, 50mM $MnCl_2$, 15% (v/v) glycerol, adjusted to pH5.8 with 0.2M acetic acid and filter sterilised), incubated on ice for 5 minutes and centrifuged at 5000rpm for 5 minutes at 4°C. The bacterial pellet was then resuspended in 0.4 volume (5ml) cold TfbII (10mM MOPS, 75mM $CaCl_2$, 10mM RbCl, 15% (v/v) glycerol, adjusted to pH6.5 with KOH and filter sterilised) and incubated on ice for 15 minutes. Aliquots of 200 µl were stored at -80°C.

2.1.4 Transformation of *E. coli*

Competent cells were thawed at room temperature and placed on ice for 10 minutes. <50ng of DNA was added to 100µl of cells, mixed gently and left on ice for 30-45 minutes. The cells were heat shocked at 37°C for 2 minutes, returned to ice for 2 minutes and 400µl of LB broth added. The cells were incubated for 1 hour at 37°C and plated out onto LB agar plates containing appropriate antibiotic (100 µg/ml ampicillin). For ligations the entire transformation was plated out, by first pelleting the cells and then resuspending them in 100 µl LB broth, otherwise, 100 µl of the total transformation was plated. The plates were inverted and incubated at 37°C overnight.

2.1.5 Small-scale plasmid preparation

A single bacterial colony was used to inoculate 5 ml LB containing 100µg/ml ampicillin. The culture was incubated overnight at 37°C with vigorous shaking. The following day, 1.5 ml of culture was removed into an eppendorf tube and cells pelleted for 1 minute at full speed in a Heraeus microfuge. The pellet was resuspended in 100 µl solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0) prior to the addition of 200 µl fresh solution II (0.2 M NaOH, 1% (w/v) SDS) and incubated on ice for 5 minutes. 150 µl cold solution III (3 M potassium acetate, 2 M acetic acid) was added, the samples vortexed and incubated on ice for a further 5 minutes. The precipitate was removed by centrifugation at full speed for 10 minutes at 4°C in a Heraeus microcentrifuge, and the supernatant transferred to a clean tube. The supernatant was extracted with phenol and chloroform as described in section 2.1 and the DNA ethanol precipitated as described in section 2.2. The DNA pellet was solublised in 30 µl water with 20µg/ml RNAse A and stored at -20°C.

2.1.6 Large-scale plasmid preparation

Large-scale plasmid preparations were performed using the QIAGEN Plasmid Midi Preparation protocol (QIAGEN) that is based on a modified alkaline lysis procedure. Briefly, 100 ml LB with 100µg/ml ampicillin was inoculated with a culture carrying the appropriate plasmid and incubated overnight at 37°C with vigorous shaking. The bacteria were pelleted by centrifugation in a Beckman M5 centrifuge with a JA-14 rotor at 5000 rpm for 10 minutes. The supernatant was discarded and the pellet resuspended in 4 ml

buffer P1. 4 ml of buffer P2 was then added, the solution mixed and incubated at room temperature for 5 minutes, after which 4 ml of chilled buffer P3 was added, mixed and incubated on ice for 15 minutes. The majority of the precipitated material was removed by centrifugation in the JA-14 rotor at 4°C for 5 minutes at 5000 rpm. The supernatant was transferred to 12 ml Beckman tubes for a further centrifugation in the JA-20.1 rotor at 4°C for 30 minutes at 15 500 rpm. The supernatant was applied to a QIAGEN-tip 100 that had been equilibrated with 4 ml of the low salt buffer, QBT. The tip was washed 2 times with 10 ml buffer QC, which is a medium salt buffer used to remove RNA, proteins, dyes and low molecular weight impurities. The DNA was then eluted with 5 ml of the high salt buffer, QF. Finally, the DNA was concentrated and desalted by precipitation with 0.7 volumes of isopropanol and pelleted at 10000rpm for 10 minutes. The DNA pellet was washed in 70% ethanol. Air dried and resuspended in 500µl of milliQ water.

For transfections, the Qiagen Endofree Plasmid Maxi-prep was used for plasmid preparations. This procedure is the same as above except the DNA is eluted from the Qiagen tip with the endofree buffer QN.

2.1.7 Restriction enzyme digestion

New England Biolabs recommendations were followed with respect to appropriate buffers for each of the enzymes. A typical reaction was carried out in a volume of 20 µl, containing 2 µl 10 x restriction buffer, 1-5 µl (approx. 1-5 µg) DNA, and 1 µl restriction endonuclease, made up to 20 µl with water. The reaction was mixed gently by flicking the side of the tube and then centrifuged briefly before being incubated at 37°C (room temperature for SmaI digestions) for 1-16 hours.

2.1.8 Conversion of 5' protruding ends to blunt ends

To the 20µl restriction enzyme digestion (containing 1-5µl DNA), 3µl 10x Klenow buffer was added and the reaction made up to 30µl with water. 1.5µl each dNTPs (2 mM) were added along with 2µl Klenow and incubated at room temperature for 15 minutes. The reaction was then terminated by the addition of 1µl 0.5 M EDTA and heat inactivated at 75°C for 10 minutes prior to gel purification.

2.1.9 Treatment of plasmid DNA with Calf Intestinal Phosphatase (CIP)

After restriction digestion DNA was phosphatase treated as follows: 10µl 10x low salt restriction enzyme buffer, the 20µl restriction digest, distilled water to 100µl and 1µl (10U) Calf Intestinal Phosphatase (CIP). The reaction was incubated at 37°C for 30 minutes. To stop the reaction, 1 µl of 0.5M EDTA, pH 8.0 was added. The CIP was heat inactivated at 70°C for 10 minutes. The DNA was then gel purified. The phosphatase treated plasmids were stored in distilled water at –20°C.

2.1.10 Ligations

Ligations were carried out in 15 µl volumes, typically containing 1.5µl 10x T4 DNA ligase buffer, 1-10 µl DNA insert, 1µl DNA vector, and 1µl T4 DNA ligase. The mix was briefly centrifuged in the Heraeus microcentrifuge and ligations incubated overnight at room temperature (approximately 18°C).

2.1.11 Agarose gel electrophoresis

Agarose gel solutions were prepared by boiling the appropriate quantity of agarose (for 1-2 % (w/v) gels) in 1x TAE buffer (50x stock: 242 g Tris, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA pH 8.0). The solution was allowed to cool to approximately 60°C before casting. The ends of the gel tray were sealed with masking tape and a comb inserted approximately 0.5cm from one end. Once set, the comb and tape were removed and the gel and tray submerged in a tank containing 1x TAE buffer. $1/6^{th}$ volume of a 6x gel-loading buffer (30% (v/v) glycerol, 0.05% (w/v) Xylene/Cyanol, 0.05% (w/v) bromophenol blue) was added to the samples, centrifuged briefly and loaded on the gel. Electrophoresis was performed at 80V with the DNA running towards the positive electrode. The gel was then stained in a solution of approx. 0.5µg/ml ethidium bromide in 1x TAE buffer for approximately 10 minutes, visualised under ultra violet light and photographed.

2.1.12 Gel purification of DNA fragments

DNA fragments were isolated from agarose gels using the QIAquick gel extraction kit (QIAGEN). Briefly, restricted DNA was separated by agarose gel electrophoresis and the ethidium bromide stained gel visualised with ultraviolet light (see previous section). The required DNA fragment was excised from the agarose gel with a clean, sharp scalpel and transferred to an eppendorf tube. Approximately 800 µl buffer QX1 was added to the gel slice and incubated at 50°C for approximately 10 minutes, until the agarose was completely dissolved. The sample was then loaded onto the QIAquick spin column and centrifuged for 1 minute at full speed in a Heraeus microcentrifuge. The column was washed once in 0.75 ml buffer PE and centrifuged in the microcentrifuge for 1 minute. Residual wash buffer was removed with a further 1 minute spin. The DNA was eluted with 30-50 µl distilled water.

2.1.13 Polymerase chain reaction

PCR reactions were carried out in 50µl volumes, containing 1x Vent buffer, 1 µl 10 mM dNTPs, 1mM sense oligonucleotide, 1mM antisense oligonucleotide, 2mM $MgSO_4$, 1 µl template, 2 µl Vent DNA polymerase. The reaction was carried out in a Perkin Elmer GeneAmp PCR System 2400 for 25 cycles with a denaturation temperature of 94°C for 45 seconds, and annealing temperature of 60°C for 30 seconds and a polymerisation temperature of 72°C for 60 seconds. The primers used are listed in Table 2.2 and the plasmid templates used are listed in Table 2.3.

2.2 Tissue Culture Techniques

2.2.1 Cell Culture

Cells were maintained at 37°C, 5% (v/v) CO_2 in a humidified incubator in RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum (Life Technologies), 20 µM 2-mercaptoethanol, 100u penicillin/streptomycin and 2 mM glutamine. BaF/3 and MC/9 cells were cultured with the addition of 5% (v/v) conditioned media from JWW3 cells as a source of murine IL-3 (see Section 2.2.15). FD-6 cells were cultured with the addition of 5% (v/v) conditioned media from X63omIL-4 as a source of murine IL-4. Cells were passaged weekly by 1:5 dilution in 5x5ml falcon dishes.

2.2.2 Preparation of Conditioned Media

JWW3 cells expressing murine IL-3 (mIL-3) or X63omIL-4 cells expressing murine IL-4 wereseeded at $1x10^5$/ml in 175 cm^2 tissue culture flasks and cultured for approximately 5 days. The media was then centrifuged for 5 minutes at 5000rpm in a Beckman M5 centrifuge with a JA-14 rotor to remove cells and cell debris and then sterilised through a 0.2 μm bottle top filter (Nalgene). Sterilised conditioned media was stored in bottles and frozen at –20 °C until required.

XTT dye reduction assays (see Section 2.2.19) were performed with Ba/F3 or FD-6 cells to test the JWW3 and X63omIL-4 conditioned media respectively. Briefly, a serial dilution series of conditioned media in RPMI 1640 was prepared across a 96 well tray and washed cells were added at 1000 per well. Cells were grown for 72 hours at 37°C before developing with XTT. The concentration of conditioned media required for maximum growth was determined and for future work, the media was supplemented with that concentration of conditioned media: typically 5% -10% v/v.

2.2.3 Transfections

10-15μg of the DNA to be transfected (pUHD10-3hygro vector containing cDNA of interest) was linearized by digestion with 3μl of FspI or PvuI overnight at 37°C in a volume of 100μl. After digestion, the DNA was purified by phenol/chloroform extraction (see section 2.1) and ethanol precipitation (see section 2.2). The DNA pellet was resuspended in 10 μl sterile electroporation buffer (25 mM HEPES, pH 7.2, 140 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 0.5% Ficoll 400, filtered through 0.2 μM). Ba/F315-1 cells were washed twice in 10 ml electroporation buffer and then pelleted at 1500 rpm for 5 minutes in a Jouan CR412 centrifuge and resuspended in electroporation buffer at $1x10^7$ cells / 0.8 ml. $1x10^7$ cells and 10-15μg of DNA were added to a sterile 0.4 cm electroporation cuvette (gap 50, BioRad) and electroporated in a BioRad Gene Pulser at 960 μF and 450V. The cells were left to stand at room temperature for 20 minutes, before being plated out into 20 ml RPMI 1640 with 5% (v/v) JWW3 conditioned medium, and 2 μg/ml tetracycline and incubated at 37°C. After 48 hours the number of viable cells was determined using trypan blue, and cells were suspended in RPMI 1640 with 5% (v/v) JWW3 conditioned medium, 2 μg/ml tetracycline, 1.5 μg/ml puromycin, and hygromycin at a concentration of 10^5 viable cells/ml. The cells were plated into 3, 96 well flat-bottomed trays (Nunc) at $2x10^4$/well.

The remaining cells were plated into a falcon 100 x 20 mm dish as a polyclonal population. After approximately 10 days, hygromycin and puromycin resistant clones from the 96 well trays were apparent and transfered into 1 ml of the same selective media in a 24 well tray (Nunc). Clones were then expanded and screened for inducible expression of the introduced cDNAs (see section 2.17). After approximately two weeks in complete selection media, selected clones were passaged in RPMI 1640 with 5% JWW3 conditioned medium and 2 μg/ml tetracycline only.

2.2.4 Screening for tetracycline-regulated expression

Hygromycin and puromycin resistant clones were expanded in RMPI 1640 with 5% (v/v) JWW3 conditioned medium in the presence of 2 μg/ml tetracycline. Cells were then washed three times in HANKS/HEPES (1:10 dilution of 10x Hank's balanced salts; 20mM HEPES, Life Technologies) buffer and pelleted at 1500 rpm for 5 minutes in a Jouan CR412 centrifuge, to remove all traces of tetracycline. The cells were resuspended at $1x10^5$ cells/ml in the presence or absence of 2μg/ml tetracycline. After 24 hours, cells were washed once in phosphate buffered saline (PBS) and cell extracts prepared at 5×10^5 cells per 50 μl solubilisation buffer (see). The amount of protein was determined by Bradford analysis (see section 2.28) and 15μg of each sample separated by SDS PAGE (see section 2.24).

2.2.5 Cell Storage

Cells from an exponentially growing dish were pelleted at 1500 rpm for 5 minutes in a Jouan CR412 centrifuge. The cell pellet was resuspended in 90% (v/v) foetal calf serum and 10% (v/v) DMSO at a concentration of greater than $2x10^6$ cell/ml. 1ml aliquots were transferred into 1ml Nunc cryotubes, wrapped in tissue, placed in a polystyrene box and frozen to -80°C. After 24 hours, the vials were transferred into liquid nitrogen.

2.2.6 XTT Assays

Serial 1:2 dilutions of growth factors in either AIM-V (a serum-free lymphocyte medium) or in RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum (Life Technologies), 20 μM 2-mercaptoethanol, 100u penicillin/streptomycin and 2 mM glutamine were performed in triplicate in 96 well flat-bottomed nunc trays. The initial

growth factor concentrations used were: IL-3, 2ng/ml; IL-4, 10ng/ml; and insulin, 1μg/ml. Cells were washed 3 times in HANKS/HEPES buffer and plated at a concentration of $1x10^5$/ml (total volume 100μL), with or without 2μg/ml tetracycline or the appropriate concentration of LY294002 (Calbiochem) or carrier (DMSO) alone. 25μl of a solution of 1mg/ml XTT and 25μM phenazine methosulphate (PMS) was added for the final 4 hours of incubation. Cells were harvested after 72 hours, and the metabolic activity of the cells determined by reduction of XTT. The soluble formazan compound was measured at 450nm on a Dynatech MR5000 plate reader.

The XTT assay system measures the bioreduction of the XTT compound to its coloured formazan compound. Thus it can be used to measure a combination of the cell number and metabolic activity of these cells, which correlates to cellular proliferation. The drawback of this assay is that a cell could be metabolically active without actual proliferation. This is in contrast to thymidine incorporation assays, which directly measure the cells use of thymidine in DNA synthesis and directly correlate to proliferation. However, in BaF/3 cells grown in response to IL-3, proliferation curves obtained by both thymidine incorporation and XTT assays were comparable (Craddock *et al.*, 1999). This indicates that in this system XTT assays are a reliable assay system with which to measure cellular proliferation.

2.2.7 Cell Viability

Cells were washed 3 times in Hanks buffer (1:10 dilution of 10x Hank's balanced salts; 20mM HEPES, Life Technologies) and plated at $1x10^5$/ml in RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum (Life Technologies), 20 μM 2-mercaptoethanol, 100u penicillin/streptomycin and 2 mM glutamine. Growth factor concentration were: IL-3, 2ng/m; IL-4, 10ng/ml; and insulin, 1μg/ml and tetracycline was used at 2μg/ml. Cells were counted in duplicate on a haemocytometer and live cells were distinguished by their exclusion of the dye trypan blue.

2.2.8 FACS Analysis

Cells were washed 3 times in Hanks/HEPES buffer and set up at $1x10^5$/ml in complete RPMI with 5% JWW3 in the presence or absence of 2μg/ml tetracycline or the appropriate concentration on LY294002. After the indicated times 10^6 cells were washed twice in PBS

then resuspended in 100µl of labelling solution (10mM Hepes/NaOH pH7.4, 140mMNaCl, 5mM$CaCl_2$, 5µg/ml propidium iodide, 20µl/ml annexin-V-FLUOS, Roche) and incubated in the dark for 15 minutes. 4ml of incubation buffer (10mM Hepes/NaOH pH7.4, 140mMNaCl, 5mM$CaCl_2$) was added before the analysis of 10 000 events per sample on a Becton-Dickenson FACS Vantage system. Annexin staining was detected on channel FL1 and propidium iodide on FL3.

2.3 Protein Chemistry Techniques

2.3.1 Cell stimulation and growth factors

Cytokine stimulations were carried out using concentrations of factor previously shown to produce maximal tyrosine phosphorylation (Welham and Schrader, 1992); IL-3, 20ng/ml; SCF, 50ng/ml (murine recombinant, purchased from R&D Systems); IL-4, 20μg/ml; GM-CSF, 5μg/ml (synthetic, gifts from Dr I. Clark-Lewis, Biomedical Research Centre, Vancover, Canada); insulin, 5μg/ml (purchased from Sigma). Before stimulation MC9 cells were starved overnight of serum and factor; cells were pelleted and resuspended in serum free media (RPMI 1640, 20μM 2-mercaptoethanol, 100u penicillin/streptomycin and 2 mM glutamine) to the original concentration. To stimulate, cells were washed 3 times in HANKS/HEPES buffer. Cells were then resuspended in serum free media at the indicated concentration and incubated at 37°C for 1 hour. After stimulation with the appropriate cytokine at 37°C, cells were pelleted for 20 seconds at 4°C in a Heraeus microcentrifuge at full speed. The supernatant was aspirated off and the cell pellets were lysed in solublisation buffer (50mM Tris-HCl pH 7.5, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 150mM NaCl, 5mM EDTA, 1mM sodium orthovanadate, 1mM sodium molybdate, 10mM sodium fluoride, 40μg/ml aprotinin, 10μg/ml soybean trypsin inhibitor, 10μg/ml leupeptin, 0.7μg/ml pepstatin). The solublised cells were pelleted at 4°C for 2 minutes and the supernatants transferred to a clean tube.

2.3.2 Immunoprecipitation of Bad

Bad was immunoprecipitated from the equivalent of $2x10^7$ cell extracts with 4μg of anti-Bad mAb (B36420, Transduction Laboratories) overnight at 4°C. Immunocomplexes were captured with 30μl of 50% (v/v) slurry of Protein-G Sepharose beads at 4°C with rotation for 1 hour. Immunoprecipitates were washed 3 times in solublisation buffer, and bound protein eluted by boiling in 1x SDS PAGE sample buffer (10%(w/v) SDS; 50%(v/v) glycerol; 0.2 M Tris-HCL, pH 6.8; bromophenol blue to colour) containing 2-mercaptoethanol.

2.2.3 SDS Polyacrylamide Gel Electrophoresis (SDS PAGE)

The Bio-Rad mini protean II system was used with a procedure similar to that of Laemlli (1970). Glass plates were cleaned with 70% ethanol and the apparatus assembled according to the manufacturers instructions. Gels were prepared as follows:

Cell extracts were fractionated on either a 7.5% polyacrylamide gel (PKB, 9E10, 4G10), or a 20% polyacrylamide gel (Bad) with a acrylamide:bisacrylamide ratio of 37.5:1. Bad immunoprecipitates were fractionated in a 12.5% polyacrylamide gel with a acrylamide:bisacrylamide ratio of 118:1 (low bis). For *in vitro* kinase assays samples were fractionated on a 15% polyacrylamide gel with a ratio of 37.5:1.

The separating gel was cast immediately after the addition of 20ul TEMED and 50 µl 10% APS by pouring 4.5ml of the acrylamide solution between the two glass plates. This was overlaid with water and allowed to polymerise (approximately 20 minutes). Once the gel had polymerised the water was aspirated off and the stacking gel (5% polyacrylaminde gel with an acrylamide to bis ratio of 37.5:1) cast over the separating gel, and a 15 well comb inserted to form wells. After polymerisation the comb was removed and the wells washed with water. The gels were the set in the tank and covered with 1x SDS running buffer (25mM Tris base, 192mM glycine, 0.1% (w/v) SDS). Samples were then loaded and run at 80V through the stacking gel followed be 150V through the separating gel. When the dye front reached the bottom of the gel, the gel was removed and placed in 1x semi-dry transfer buffer (39mM glycine, 48mM Tris base, 0.0375% (w/v) SDS, 20% methanol).

2.3.4 Immunoblotting

A Pharmacia LKB NovaBlot was used to transfer proteins by semi-dry transfer to nitrocellulose membranes. A sandwich was formed by placing 4 pieces of dampened (semi-dry transfer buffer, see Section 2.2.3) 3 MM Whatman filter paper on the positive (lower) electrode, followed by the wet nitrocellulose, the gel and a further 4 pieces of damp filterpaper. The negative (upper) electrode was positioned on top and the gel transferred at 0.8 mA/cm^2 for 1 hour. After transfer the membranes were rinsed in water, and stained with Ponceau S to allow the position of the molecular weight standard to be marked and the transfer efficiency and protein loading to be assessed. The stain was removed by washing in Tris-buffered saline (TBS (20mM Tris-HCL, pH 7.5; 150 mM NaCL). Membranes were blocked in blocking buffer (5% (w/v) BSA, 1% (w/v) ovalbumin, 0.05% sodium azide solution in TBS) for at least 1 hour. Primary antibodies were prepared in a 1:5 dilution of

blocking buffer and used at the concentrations outlined in Table 2.1. The blots were incubated overnight with all primary antibodies except for 4G10 and 9E10, which were incubated for 3 hours. After incubation with primary antibody, blots were washed 1 x 10 minutes in TBS, 3 x 10 minutes in TBSN (TBS with 0.05% (v/v) NP-40), and 1 x 10 minutes in TBS. Blots were then incubated for 1-2 hours with the secondary antibody. Both goat anti-rabbit and goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (Dako) were used at a concentration of 0.05 μg/ml (1:20 000 dilution) in TBSN. Secondary antibody was washed from the blots as for the primary antibody with a final 10 minute wash in TBS. After the final washing, the blots were placed in a clean container and developed in enhanced chemiluminescence (ECL) or enhanced chemilluminescence plus (ECLplus) (AmershamPharmacia) for 1 minute (ECL) or 5 minutes (ECLplus). Kodak XAR-5 film was used for detection of ECL signals and films were developed in a Fuji X-ray Processor RG2. The films were scanned using a Mustek 1200CU scanner for presentation in this thesis.

2.3.5 Stripping of Blots

To strip immunoblots completely of protein, nitrocellulose membranes were incubated in stripping solution (62.5 mM Tris-HCl, pH 6.7, 2% (w/v) SDS, 100 mM β-mercaptoethanol) at 55°C for 60 minutes with periodic agitation. Blots were extensively washed in TBSN prior to being blocked in blocking buffer and probed with antibody as described above.

2.3.6 *In vitro* kinase assays

Cells were stimulated as described above. PKB was immunoprecipitated from cell lysates with 1μg anti-PKB (Santa Cruz) or 5μg 9E10 for 1 hour at 4°C. Immunocomplexes were captured with 30μl of Protein G Sepharose at 4°C with rotation for 1 hour. Beads were washed twice in solublisation buffer, twice in LiCl wash buffer (500mM LiCl; 100mM Tris HCl, pH 7.5; 1mM EDTA) followed by one wash in kinase buffer (50mM Tris HCl, pH 7.5; 10mM $MgCl_2$; 1mM DTT). Kinase assays were performed by suspending beads in kinase buffer containing 2.5μg histone2B, 0.5μM protein kinase inhibitor, 50μM ATP, 3μCi ATPγ32P at room temperature for 30 minutes. Reactions were stopped by the addition of 5x SDS PAGE sample buffer containing 2 mercaptoethanol followed by boiling for 5 minutes. Samples were fractionated and immunoblottted as described in section 2.3.5.

After transfer membranes were cut in half, the upper part was immunoblotted for PKB (see Section 2.3.4) and the lower part wrapped in cling film and subjected to autoradiography. Kodak XAR-5 film was used for detection and films were developed in a Fuji X-ray Processor RG2. After autoradiography, the H2B bands were excised, placed into 5ml of scintilation fluid and the counted on a Wallac 1215 Rackbeta liquid scintillation counter.

2.3.8 Bradford Protein Estimations

BSA of known concentration or samples of cell lysates were added to 0.5 ml aliquots of water in Eppendorf tubes. 0.5 ml of the Bradford reagent (200 mg Coomassie Blue G-250 in 200 ml 85% (v/v) H_3PO_4, made up to 1.0 L with water and then filtered) was added to each tube and vortexed. 100 µl was then added to wells in a 96 well round bottom tray (Falcon) and the optical density at 595 nm determined on a Dynatech MR5000 plate reader. A standard curve was constructed from the BSA standards and the protein concentrations of the cell lysates determined. This procedure was adapted from Bradford (1976).

2.4 Materials

2.4.1 Antibodies

Table 2.4.1

Antibodies used for Blotting and Precipitation

Antibody	Source	Usage
4G10 (monoclonal, anti-phosphotyrosine)	Upstate Biology Inc (cat. # 05-321)	Blot: 0.1 μg/ml
9E10 (monoclonal anti-myc-tag)	ATCC hybridoma line	Blot: 0.5 μg/ml I.P.: 5 μg/sample
anti-phosphoPKBSer^{473}	New England Biolabs (cat. # 9271)	Blot: 1:1000
anti-phosph-PKBThr^{308}	New England Biolabs (cat. # 9275)	Blot: 1:1000
anti-PKB	New England Biolabs (cat. # 9272)	Blot: 1:1000
PKB (PHdomain)	Upstate Biology Inc (cat #06-885)	Blot: 1μg/ml
anti-PKB C20	Santa Cruz (cat. # sc-1618)	I.P.: 1μg
anti-Bad	Santa Cruz (cat. # sc-943)	Blot: 0.5 μl/ml
anti-Bad	Transduction Labs (cat. # B36420)	I.P.: 4μg/ml
anti-phospho-BadSer^{112}	New England Biolabs (cat. # 9290)	Blot: 1:1000
anti-phospho-BadSer^{136}	New England Biolabs (cat. # 9295)	Blot: 1:1000
Anti-phospho-Bad Ser^{136}	Upstate Biology Inc (cat. # 06-799-MN)	Blot: 2μg/ml
anti-Bad	R&D (cat. # AF819)	Blot: 1μg/ml
goat-anti-rabbit-HRP conjugated	Dako	1:20000
goat-anti-mouse-HRP conjugated	Dako	1:20000

2.4.2 Primers

Table 2.4.2
Primers used for PCR

Insert	Forward primer	Reverse primer
wtPKB	cacggatccatccatgaacgacgt	cgggatcctcaggccgtc
kdPKB	cacggatccatccatgaacgacgt	cgggatcctcaggccgtc
cxPKB	cacggatccatccatgaacgacgt	tggatccttacataattacacaccttgt
gagPKB	tctggatccatgggacagac	cgggatcctcaggccgtc

2.4.3 Plasmids

Table 2.4.3

Plasmids used for Cloning and Transfection

Vector	Use	Source
PSG5gagPKB	Source of gagPKB construct	van Weeren *et al.*, 1998, Burgerring and Coffer, 1995
PSG5kdPKB	Source of kdPKB construct	
PSG5PKB	Source of wtPKB construct	
PSG5cxPKB	Source of cxPKB construct	
pBS.Nmyc2	Source of N-terminal myc-tag	Craddock *et al.*, 1999
pUHD10-3(hygro)	Hygromycin resistant response plasmid	Mui *et al.*, 1996
pUHD10-3(neo)	G418 resistant response plasmid	
pUHD15-1(puro)	Regulator plasmid	

2.4.4 Reagents

Table 2.4.4

Reagents used and their Suppliers

Reagents	Supplier*
1 Kb DNA ladder	Life Technologies
β-mercoptoethanol	Bio-Rad
[γ-^{32}P] ATP	NEN
Acetic acid (glacial)	BDH
30% Acrylamide/Bis solution 37.5:1	Bio-Rad
agarose (electrophoresis grade)	Life Technologies
AIM-V	Life Technologies
Albumin, Chicken Egg (Grade III)	Sigma
Ammonium persulphate	Fisons Scientific
Ampicillin	Sigma
Annexin V Fluos	Roche
Aprotinin	Roche
Bactoagar	Difco Laboratories
Bactotryptone	Difco Laboratories
Bovine Serum Albumin (BSA)	Roche
Bromophenol Blue	Fisons Scientific
Calcium chloride ($CaCl_2$)	Fisons Scientific
Calf intestinal phosphatase (CIP)	New England Biolabs
Chloroform	BDH
Diaminoethanetetra acetic acid (EDTA)	Sigma
Dideoxy nucleotide triphosphates (dNTPs)	New England Biolabs
Dimethyl sulphoxide (DMSO)	Sigma
ECL	Amersham
ECL plus	Amersham
Ethanol (99.6%)	BDH
Fetal calf serum	Autogen Bioclear
Ficoll 400	SIGMA
Geneticin (G418)	Life Technologies
Glutathione Sepharose 4B	Pharmacia Biotech
Glycerol	SIGMA
Glycine	SIGMA
GM-CSF	Dr Ian Clarke-Lewis
HANKS buffered saline (10x)	Life Technologies
HEPES	Life Technologies
Histone 2B	Sigma
IL-3	R & D Systems
IL-4	Dr Ian Clarke Lewis
Insulin	Sigma
Klenow	New England Biolabs

L-Glutamine (100X)	Life Technologies
Leupeptin	Sigma
Lysozyme	Sigma
Magnesium chloride ($MgCl_2$)	Sigma
Magnesium sulphate (50X)	New England Biolabs
Manganese chloride ($MnCl_2$)	Sigma
Methanol	BDH
MOPS	Sigma
Nitrocellulose	BDH
NP-40	Sigma
Oligonucleotides	Pharmacia Biotech
Penecillin-Streptomycin	Life Technologies
Pepstatin A	Roche
Phenazine methosulphate (PMS)	Sigma
Phenol (buffer-saturated)	Life Technologies
Phenylmethylsulphonyl fluoride (PMSF)	Sigma
Phosphate Buffered Saline (PBS)	Life Technologies
Phosphoric Acid (85% (v/v)) H_3PO_4	Sigma
Ponceau S	Sigma
Potassium acetate (KAc)	Sigma
Potassium Chloride (KCl)	Sigma
Potassium Hydoxide (KOH)	Sigma
Propidiun Iodide	Sigma
Protein-G Sepharose	Pharmacia Biotech
Puromycin	Calbiochem
Recombinant mouse IL-3 (rmIL-3)	R&D Systems
Restriction Endonuleases	New England Biolabs
RNase A	Roche
RPMI 1640	Life Technologies
Rubidium chloride	Sigma
SCF	R & D Systems
SDS-PAGE standards (broad range)	Bio-Rad
Sodium acetate (NaAc)	Sigma
Sodium azide	Fisons Scientific
Sodium chloride (NaCl)	Sigma
Sodium dodecyl sulphate (SDS)	BDH
Sodium hydroxide (NaOH)	Fisons Scientific
Sodium fluoride (NaF)	Sigma
Sodium molybdate	BDH
Sodium orthovanadate	Sigma
Soybean Trypsin Inhibitor	Sigma
T4 DNA ligase	New England Biolabs
Tetracycline	Sigma
Tetramethylethylenediamine (TEMED)	Bio-Rad
Trizma base (Tris)	Sigma
Vent DNA polymerase	New England Biolabs

X-ray film (XAR-5)	Kodak
XTT	Sigma
Xylene/Cyanol	Sigma
Yeast extract	Difco Laboratories

***Full name and location of Suppliers:**

Dr Ian Clarke-Lewis, BRC, Vancover, Canada

Amersham Pharmacia Biotech, Herts, U.K.

Autogen Bioclear, Wilts, U.K.

BDH Chemicals Ltd., Poole, U.K.

Bio-Rad, Richmond, California, USA

Calbiochem, Nottingham, U.K.

Dako, Denmark

Difco Laboratories, Detroit, Michigan, USA

Eastman Kodak Company, Rochester, NY, USA

Fisons Scientific, Leiscester, U.K.

Life Technologies Ltd., Paisley, U.K.

NEN Life Science Products, Holland

New England Biolabs Inc., MA, USA

R&D Systems Europe Ltd., Abringdon, U.K.

Roche, East Sussex, UK

Sigma Chemicals, Poole, U.K.

Chapter 3

Comparison of PKB Signalling in BaF/3, FD6 and MC9 Cells

3.1 Introduction

When this study was initiated the effects of cytokine stimulation on PKB were largely unknown. PKB was first shown to be activated downstream of PI3K in response to PDGF (Franke *et al.*, 1995; Burgering and Coffer, 1995), insulin (Cross *et al.*, 1995; Burgering and Coffer, 1995), EGF and bFGF (Burgering and Coffer, 1995) and the IL-3 stimulated activation of PKB had been demonstrated in FL5.12 (del Peso *et al.*, 1997) and 32D (Datta *et al.*, 1997; Songyang *et al.*, 1997; Songyang *et al.*, 1997) cells (Datta *et al.*, 1997; Songyang *et al.*, 1997) but the effects of SCF and IL-4 had not been investigated. PKB had been implicated in the PI3K mediated survival and proliferative pathway (Dudek *et al.*, 1997; Kauffman-Zeh *et al.*, 1997; Kennedy *et al.*, 1997; Khwaja *et al.*, 1997; Songyang *et al.*, 1997), but it was unclear whether PKB activation was sufficient to provide a survival signal or indeed necessary for survival and proliferation.

Haemopoietic cell lines are dependent on the presence of the appropriate cytokine(s) for their continued growth and survival. In the absence of cytokine, cells cease to proliferate and undergo programmed cells death or apoptosis. This feature makes them a good model in which to investigate the signal transduction mechanisms leading to proliferation and apoptosis.

Therefore, the objective of this chapter was to investigate the stimulation of haemopoietic cells by IL-3, IL-4 and SCF with respect to the activation of PKB and relate this to the functional responses of proliferation and/or survival. Three haemopoietic cell lines were utilised:

- MC9, an IL-3 dependent mast cell line which is responsive to IL-4, SCF, insulin and GM-CSF (Nabel *et al.*, 1981).
- FD-6, a myeloid progenitor cell line which is dependent on IL-4 and responsive to IL-3, insulin and GM-CSF (Welham *et al.*, 1994a).
- BaF/3 a pre B-cell line that is IL-3 dependent and responds to IL-4 and insulin (Palacios and Steinmetz, 1985).

3.2 Cytokine induced activation of PKB.

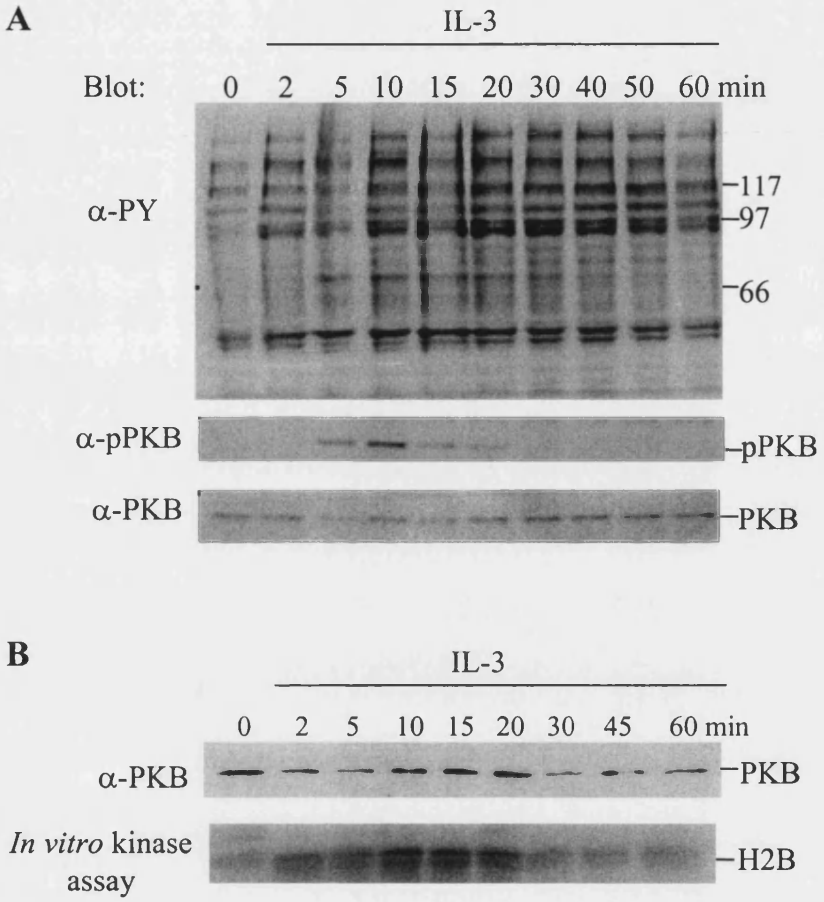
The first objective was to determine whether PKB is activated in response to various cytokines in BaF/3, FD-6 and MC9 cell lines. Extensive time-course analyses were performed and the activation of PKB assessed firstly by examining the phosphorylation of Ser^{473} using phospho-specific antibodies and secondly by directly examining the kinase activity of PKB by looking at the incorporation of $\gamma^{32}P$ (phosphate group) into the substrate histone2B. Ser^{473} is one of two sites on PKB that is phosphorylated in the active form (Alessi *et al.*, 1996a) hence looking directly at the phosphorylation of this site gives an indication of the activation state of PKB. Histone2B was used as the substrate in *in vitro* kinase assays as it is phosphorylated by active PKB at Ser^{36}, which lies within a PKB consensus sequence (Alessi *et al.*, 1996b). To ensure that the cytokine-induced stimulation of cells was efficient, the overall level of tyrosine phosphorylation was also examined. Duplicate gels were run and these were immunoblotted with 4G10 antibody, which recognises phosphorylated tyrosine residues.

3.3 Kinetics of PKB activation in FD6 Cells

FD6 cells are dependent on either IL-3 or IL-4 for their continued growth and survival. Given that PKB was thought to have a role downstream of PI3K in proliferation and survival, it was of interest to see what effect the stimulation of FD6 cells with IL-3 and IL-4 had on PKB activation. Both IL-3 (Figure 3.1A, middle panel) and IL-4 (Figure 3.2A, middle panel) induce the phosphorylation of PKB at Ser^{473} as determined by immunoblotting with an antibody specific to the phosphorylated form of PKB-Ser^{473}. Maximum phosphorylation was observed at 10 minutes and phosphorylation had returned to basal levels by 30 minutes with both IL-3 and IL-4. The IL-3 and IL-4 induced phosphorylation of Ser^{473} mimicked the patterns of tyrosine phosphorylation induced by these factors (Figure 3.1A and 3.2A, upper panels).

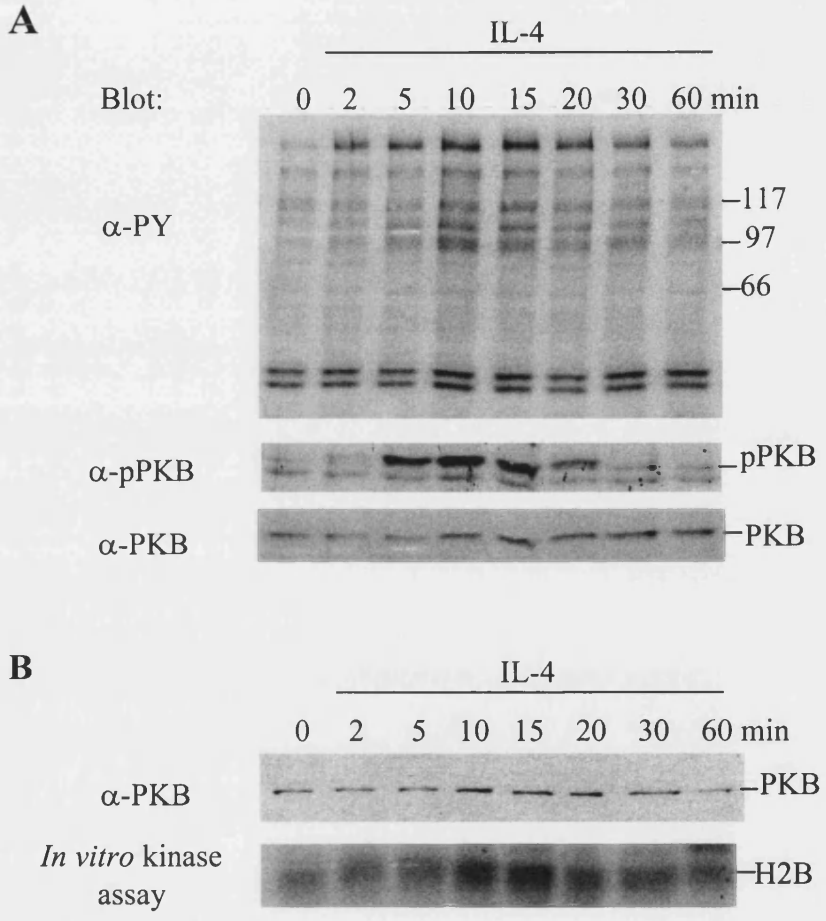
Ser^{473} is one of two sites on PKB phosphorylated in its activated form. Therefore, studying the phosphorylation at this site gives an indication of the activity of PKB but does not measure its activity directly. *In vitro* kinase assays were performed to look directly at the activity of PKB and to confirm that Ser^{473} phosphorylation mirrors the activation state of PKB. Both IL-3 (Figure 3.1B) and IL-4 (Figure 3.2B) induced the activation of PKB in FD6 cells and the kinetics of activation followed the kinetics of Ser^{473} phosphorylation. This confirms firstly that PKB is activated in response to IL-3 and IL-4 in FD6 cells and secondly that the phosphorylation status of Ser^{473} is a good indicator of the activity of PKB.

Figure 3.1
IL-3-Induced Activation of PKB in FD-6 Cells



FD-6 cells were starved of serum and factor for 1hr before stimulation with 20ng/ml rmIL-3 for the indicated times. **(A)** Cells were lysed and whole cell extracts ($8x10^5$ cell equivalents) were separated on SDS-PAGE and transferred to nitrocellulose. These were immunoblotted for α-phospho-tyrosine (α-PY, upper panel) or α-phosph-Ser^{473} PKB (α-pPKB, middle panel). The PKB blots were stripped and reprobed with α-PKB (lower panel). **(B)** Cells were lysed (10^7 cell equivalents) and immunoprecipitated with α-PKB. *In vitro* kinase assays were performed using histone2B (H2B) as the substrate and the products were separated by SDS-PAGE and transferred to nitrocellulose. The membranes were cut in half, the upper immunoblotted with α-PKB (upper panel) and the lower subjected to autoradiography (lower panel). This experiment is representative of (A) 2 and (B) 3 independent experiments. The experiments shown in (A) and (B) are not performed on cell extracts from the same stimulation.

Figure 3.2
IL-4-Induced Activation of PKB in FD-6 Cells

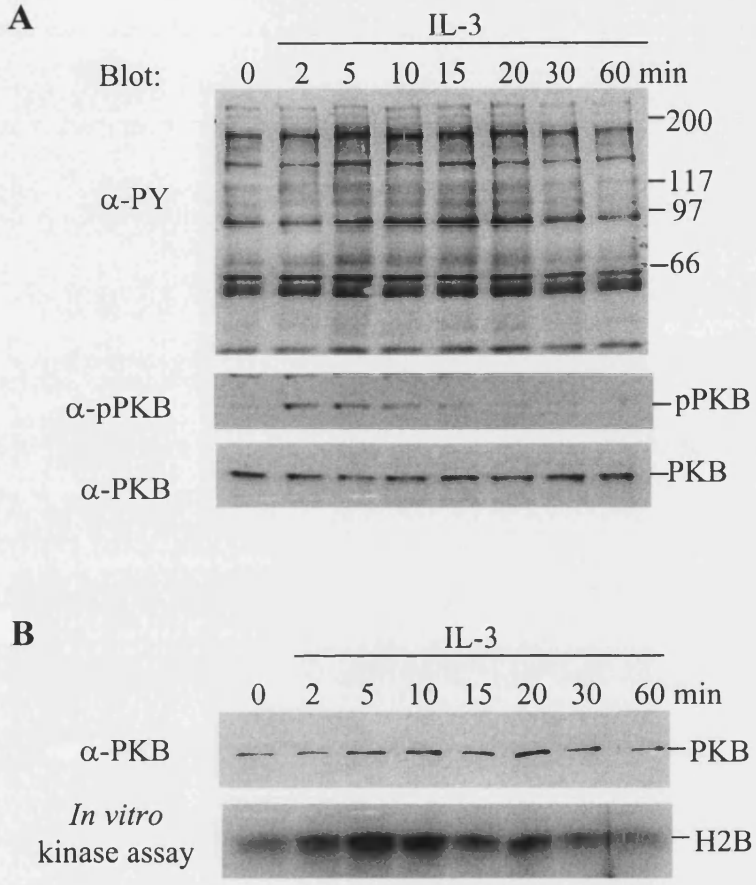


FD-6 cells were starved of serum and factor for 1hr before stimulation with 20μg/ml synthetic IL-4 for the indicated times. **(A)** Cells were lysed and whole cell extracts ($8x10^5$ cell equivalents) were separated on SDS-PAGE and transferred to nitrocellulose. These were immunoblotted for α-phospho-tyrosine (α-PY, upper panel) or α-phosph-Ser^{473} PKB (α-pPKB, middle panel). The PKB blots were stripped and reprobed with α-PKB (lower panel). **(B)** Cells were lysed (10^7 cell equivalents) and immunoprecipitated with α-PKB. *In vitro* kinase assays were performed using histone2B (H2B) as the substrate and the products were separated by SDS-PAGE and transferred to nitrocellulose. The membranes were cut in half, the upper immunoblotted with α-PKB (upper panel) and the lower subjected to autoradiography (lower panel). This experiment is representative of (A) 3 and (B) 4 independent experiments. The experiments shown in (A) and (B) are not performed on cell extracts from the same stimulation.

3.4 Kinetics of PKB activation in MC9 Cells

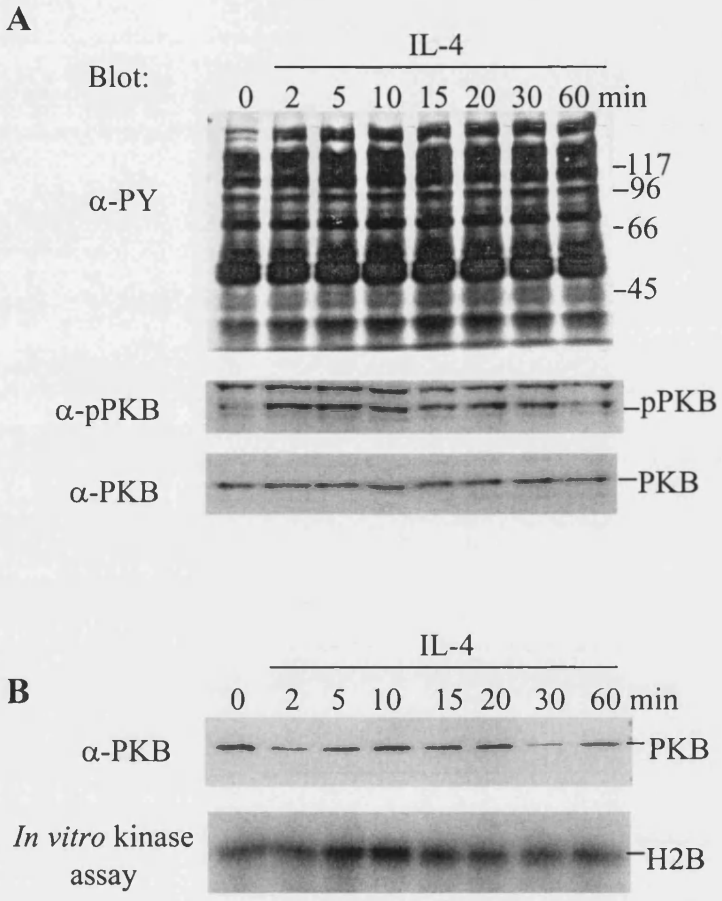
MC9 cells are dependent on IL-3 for their continued growth and survival and are also responsive to IL-4 and SCF. To examine the role that PKB may play in these functions the activity of PKB in response to IL-3, IL-4 and SCF was examined. Firstly, the phosphorylation of Ser^{473} was examined. Phosphorylation induced by IL-3 reached a maximum by 2-5 minutes and was relatively short-lived, being back to basal levels by 20-30 minutes (Figure 3.3A). IL-4 induced phosphorylation of Ser^{473} was more prolonged with high levels of phosphorylation still observed at 30 minutes (Figure 3.4A, middle panel). The SCF induced phosphorylation was very rapid, high levels were observed at 1 minute, it reached a peak at 2 minutes and was back to basal levels by 30 minutes (Figure 3.5A, middle panel). As seen in the FD6 cell line the phosphorylation of Ser^{473} closely followed the pattern of overall tyrosine phosphorylation (Figures 3.3-3.5A, upper and middle panels). To confirm that Ser^{473} phosphorylation correlates with an increased activity of PKB, *in vitro* kinase assays were performed (Figure 3.3B, 3.4B, 3.5B). These confirmed that all three growth factors induce the activation of PKB in MC9 cells, with similar kinetics observed with both IL-3 and SCF. Interestingly, IL-4-induced a sustained phosphorylation at Ser^{473} with near maximal phosphorylation observed at 30 minutes but this was not reflected in the kinase assays where the activity observed 30 minutes after stimulation was markedly less than maximal (Figure 3.4A, middle panel and 3.4B, lower panel).

Figure 3.3
IL-3-Induced Activation of PKB in MC9 Cells



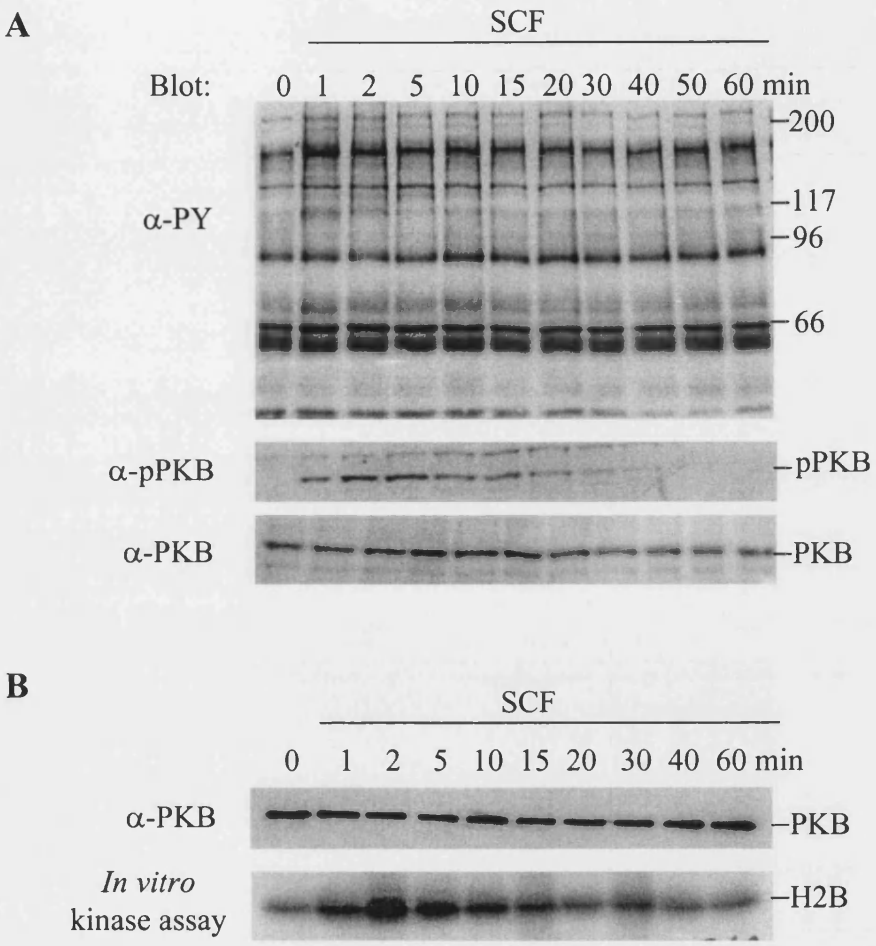
MC9 cells were starved of serum and factor for 1hr before stimulation with 20ng/ml rmIL-3 for the indicated times. **(A)** Cells were lysed and whole cell extracts ($8x10^5$ cell equivalents) were separated on SDS-PAGE and transferred to nitrocellulose. These were immunoblotted for α-phospho-tyrosine (α-PY, upper panel) or α-phosph-Ser^{473} PKB (α-pPKB, middle panel). The PKB blots were stripped and reprobed with α-PKB (lower panel). **(B)** Cells were lysed (10^7 cell equivalents) and immunoprecipitated with α-PKB. *In vitro* kinase assays were performed using histone2B (H2B) as the substrate and the products were separated by SDS-PAGE and transferred to nitrocellulose. The membranes were cut in half, the upper immunoblotted with α-PKB (upper panel) and the lower subjected to autoradiography (lower panel). This experiment is representative of (A) 3 and (B) 2 independent experiments. The experiments shown in (A) and (B) are not performed on cell extracts from the same stimulation.

Figure 3.4
IL-4-Induced Activation of PKB in MC9 Cells



MC9 cells were starved of serum and factor for 1hr before stimulation with 20μg/ml synthetic IL-4 for the indicated times. **(A)** Cells were lysed and whole cell extracts ($8x10^5$ cell equivalents) were separated on SDS-PAGE and transferred to nitrocellulose. These were immunoblotted for α-phospho-tyrosine (α-PY, upper panel) or α-phosph-Ser^{473} PKB (α-pPKB, middle panel). The PKB blots were stripped and reprobed with α-PKB (lower panel). **(B)** Cells were lysed (10^7 cell equivalents) and immunoprecipitated with α-PKB. *In vitro* kinase assays were performed using histone2B (H2B) as the substrate and the products were separated by SDS-PAGE and transferred to nitrocellulose. The membranes were cut in half, the upper immunoblotted with α-PKB (upper panel) and the lower subjected to autoradiography (lower panel). This experiment is representative of (A) 2 and (B) 3 independent experiments. The experiments shown in (A) and (B) are not performed on cell extracts from the same stimulation.

Figure 3.5
SCF-Induced Activation of PKB in MC9 Cells



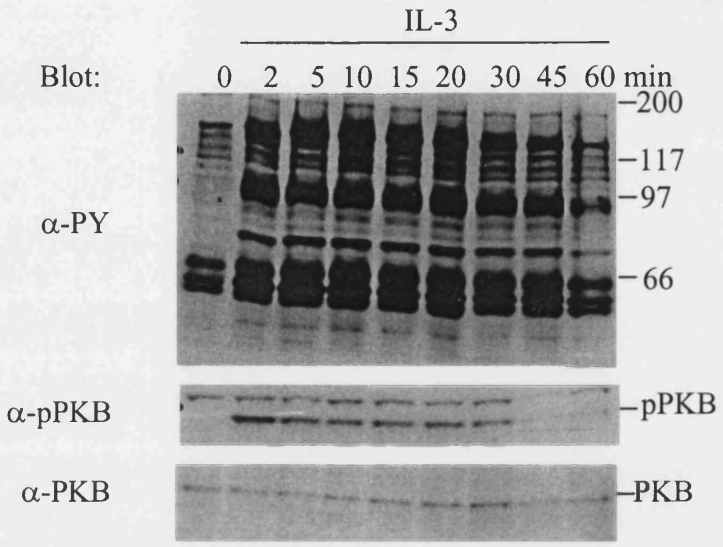
MC9 cells were starved of serum and factor for 1hr before stimulation with 50ng/ml SCF for the indicated times. **(A)** Cells were lysed and whole cell extracts ($8x10^5$ cell equivalents) were separated on SDS-PAGE and transferred to nitrocellulose. These were immunoblotted for α-phospho-tyrosine (α-PY, upper panel) or α-phosph-Ser^{473} PKB (α-pPKB, middle panel). The PKB blots were stripped and reprobed with α-PKB (lower panel). **(B)** Cells were lysed (10^7 cell equivalents) and immunoprecipitated with α-PKB. *In vitro* kinase assays were performed using histone2B (H2B) as the substrate and the products were separated by SDS-PAGE and transferred to nitrocellulose. The membranes were cut in half, the upper immunoblotted with α-PKB (upper panel) and the lower subjected to autoradiography (lower panel). This experiment is representative of (A) 4 and (B) 2 independent experiments. The experiments shown in (A) and (B) are not performed on cell extracts from the same stimulation.

3.5 Kinetics of PKB activation in BaF/3 Cells

In BaF/3 cells, a cell line that is dependent of IL-3 for its continued growth and survival, stimulation with IL-3 resulted in the rapid phosphorylation of PKB at Ser^{473} (Figure 3.6, middle panel). Maximum phosphorylation was observed at 2 minutes and levels were still high at 30 minutes. The IL-3 induced phosphorylation of Ser^{473} closely mimicked the overall pattern of tyrosine phosphorylation induced with this factor.

In the three cell lines examined all the cytokines tested were able to induce the phosphorylation of Ser^{473} and histone2B indicating that PKB is activated (Figures 3.1-3.6). The kinetics of PKB activation varied according to each cytokine and cell line but followed closely to the patterns of total cellular tyrosine phosphorylation. These cell lines are dependent on particular cytokines for their continued growth and survival, and they will also respond to stimulation by others. Given that PKB has been reported to be involved in growth and survival, it is interesting that all the cytokines tested were able to stimulate the activation of PKB in these cells yet not all of these cytokines are able to support their long-term survival: IL-3 is required for the long-term growth and survival of BaF/3 and MC9 cells, while IL-3 or IL-4 is needed for the survival of FD-6 cells.

Figure 3.6
IL-3–induced phosphorylation of PKB in BaF/3 cells



BaF/3 cells were starved of serum and factor for 1hr before stimulation with 20ng/ml rmIL-3 for the indicated times. For immunoblotting cells were lysed and whole cell extracts ($8x10^5$ cell equivalents) were separated on SDS-PAGE and transferred to nitrocellulose. These were immunoblotted for α-phosphotyrosine (α-PY, upper panel) or α-phosph-Ser^{473} PKB (α-pPKB, middle panel). The PKB blots were stripped and reprobed with α-PKB (lower panel). This experiment is representative of 3 independent experiments.

3.6 Dose Response Analyses

The concentration of growth factor used in the above experiments was that previously found to induce maximal tyrosine phosphorylation (Welham and Schrader, 1992). To confirm that this concentration also induces maximal PKB phosphorylation dose response analyses were performed. For all these experiments, stimulations were carried out for the length of time shown above to induce maximal PKB phosphorylation in the particular cell line examined.

3.7 Dose Response Analysis in FD6 Cells

In FD-6 cells both IL-3 (Figure 3.7A) and IL-4 (Figure 3.7B) activated PKB in a dose responsive manner, which mimicked the total cellular tyrosine phosphorylation induced by each cytokine. Maximal PKBSer^{473} phosphorylation was observed at 20ng/ml using recombinant IL-3 and 20μg/ml using synthetic IL-4. These concentrations were used in subsequent experiments.

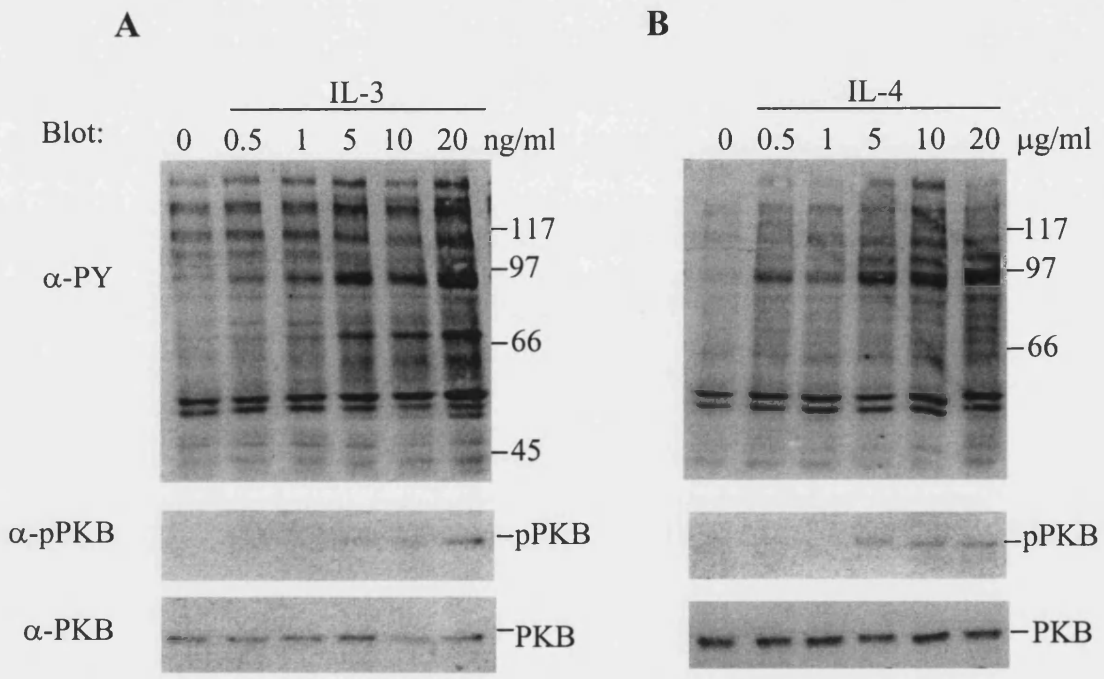
3.8 Dose Response Analysis in MC9 Cells

In SCF-stimulated MC9 cells maximal Ser^{473} phosphorylation occurred between 5 and 50ng/ml, while maximal tyrosine phosphorylation was seen at 50ng/ml SCF (Figure 3.8A). In MC9 cells stimulated with synthetic IL-4 maximal Ser^{473} and tyrosine phosphorylation was observed at 20μg/ml (Figure 3.8B). When cells were stimulated with IL-3 maximal phosphorylation was observed at 10-20ng/ml (Figure 3.9). In subsequent experiments MC9 cells were stimulated with 10ng/ml IL-3, 50ng/ml SCF and 20μg/ml IL-4.

3.9 Dose Response Analysis in BaF/3 Cells

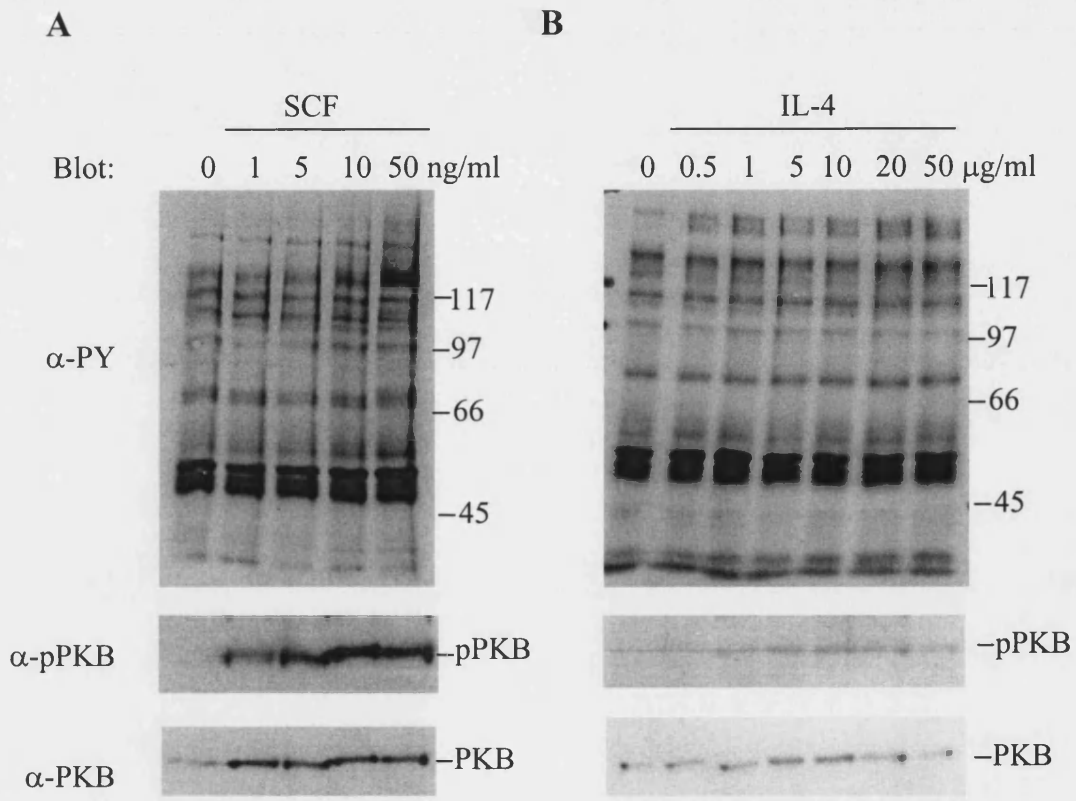
In BaF/3 cells IL-3 activated PKB in a dose-responsive manner (Figure 3.10). Maximal tyrosine and Ser^{473} phosphorylation were observed at 10-20ng/ml IL-3 and 10ng/ml was used in subsequent experiments.

Figure 3.7
Dose-Response analyses in FD-6 Cells



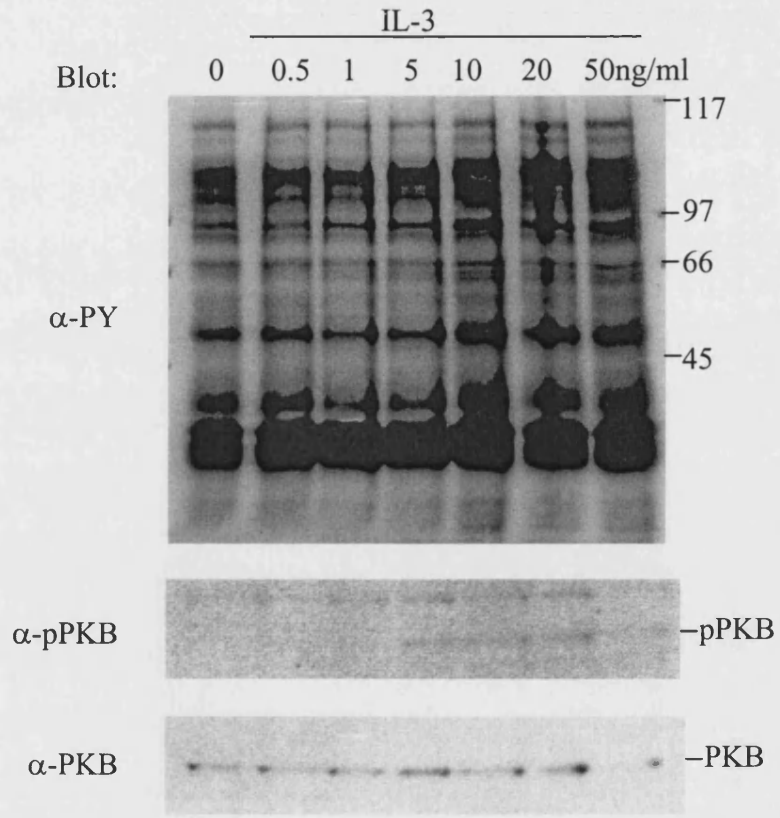
FD-6 cells were starved of serum and factor for 1hr prior to stimulation with the appropriated dose of cytokine for 5 min (IL-3), 10 min (IL-4). Cells were lysed and whole cell extracts ($8x10^5$ cell equivalents) separated by SDS-PAGE and transferred to nitrocellulose. These were immunoblotted with α-phospho-tyrosine (α-PY, upper panels) or α-phospho-Ser^{473} PKB (α-pPKB, middle panels). The PKB blots were stripped and reprobed with α–PKB (lower panels). These experiments are representative of (A) 4 and (B) 4 independent experiments.

Figure 3.8
Dose-Response Analysis of MC9 Cells to IL-4 and SCF



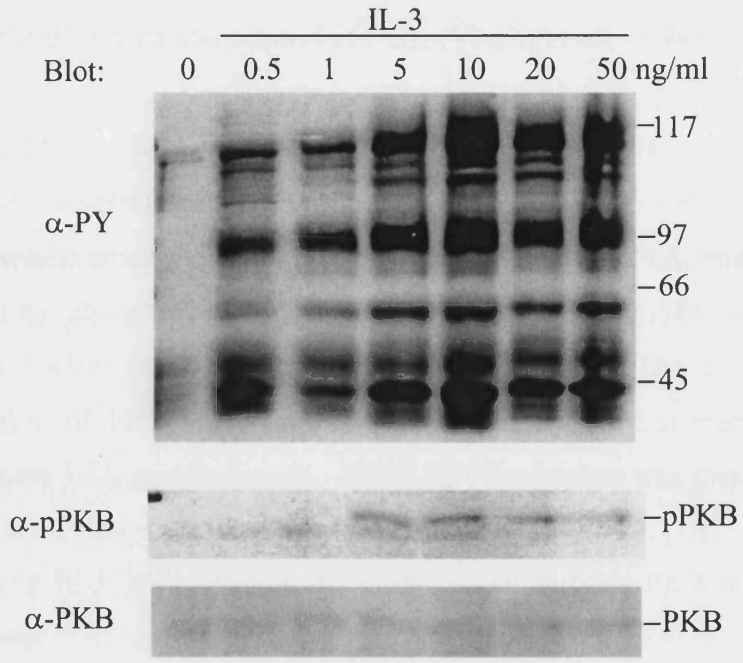
MC9 cells were starved of serum and factor for 1hr prior to stimulation with the appropriated dose of cytokine for 5 min. Cells were lysed and whole cell extracts ($8x10^5$ cell equivalents) separated by SDS-PAGE and transferred to nitrocellulose. These were immunoblotted with α-phospho-tyrosine (α-PY, upper panels) or α-phospho-Ser^{473} PKB (α-pPKB, middle panels). The PKB blots were stripped and reprobed with α–PKB (lower panels). The experiment shown in (A) was performed once, while the experiment shown in (B) is representative of 3 independent experiments.

Figure 3.9
Dose Response Analysis of MC9 Cells to IL-3



MC9 cells were starved of serum and factor for 1hr prior to stimulation with the appropriated dose of IL-3 for 5 min. Cells were lysed and whole cell extracts ($8x10^5$ cell equivalents) separated by SDS-PAGE and transferred to nitrocellulose. These were immunoblotted with α-phospho-tyrosine (α-PY, upper panel) or α-phospho-Ser^{473} PKB (α-pPKB, middle panel). The PKB blots were stripped and reprobed with αPKB (lower panel). This experiment is representative of 2 independent experiments.

Figure 3.10
Dose response analysis of BaF/3 Cells to IL-3



BaF/3 cells were starved of serum and factor for 1hr prior to stimulation with the appropriated dose of cytokine for 5 min. Cells were lysed and whole cell extracts ($8x10^5$ cell equivalents) separated by SDS-PAGE and transferred to nitrocellulose. These were immunoblotted with α-phospho-tyrosine (α-PY, upper panel) or α-phospho-Ser^{473} PKB (α-pPKB, middle panel). The PKB blots were stripped and reprobed with α–PKB (lower panel). This experiment was performed once.

3.10 Effect of PI3K Inhibition on PKB activation

Activation of PKB has been shown to be mediated through PI3K in a number of systems (Dudek *et al.*, 1997; Kauffman-Zeh *et al.*, 1997; Kennedy *et al.*, 1997; Khwaja *et al.*, 1997; Songyang *et al.*, 1997). Hence it was of interest to determine what effect inhibition of PI3K would have on PKB activation in these haemopoietic cells. The time-points and concentration of factor used in these experiments were those that induced maximal PKB activity, as determined in the previous experiments (Figures 3.1-3.10). Cells were treated with the pharmacological PI3K inhibitor LY294002 prior to cytokine stimulation and the phosphorylation of histone2B and PKBSer^{473} examined. LY294002 is a reversible inhibitor, which competes for binding to the active ATP binding site in the p110 catalytic subunit of PI3K (Vlahos *et al.*, 1994).

3.11 Effect of PI3K Inhibition on PKB activation in FD-6 Cells

In FD-6 cells, pre-treatment with 10μM LY294002 completely abrogated the PKBSer^{473} phosphorylation induced by IL-3 and IL-4 (Figure 3.11A, middle panel). It greatly reduced the phosphorylation induced by treatment with GM-CSF and reduced that induced by insulin. In FD-5 cells (a different sub-clone to FD-6 cells) the insulin-induced activation of PKB was completely inhibited by pre-treatment with 30μM LY294002 (Figure 3.12, middle panel). When PKB activation was measured directly using *in vitro* kinase assays similar results were obtained (Figure 3.11B). This indicates that in FD-6 cells IL-3, IL-4, insulin and GM-CSF all activate PKB through a PI3K mediated pathway.

3.12 Effect of PI3K Inhibition on PKB activation MC9 Cells

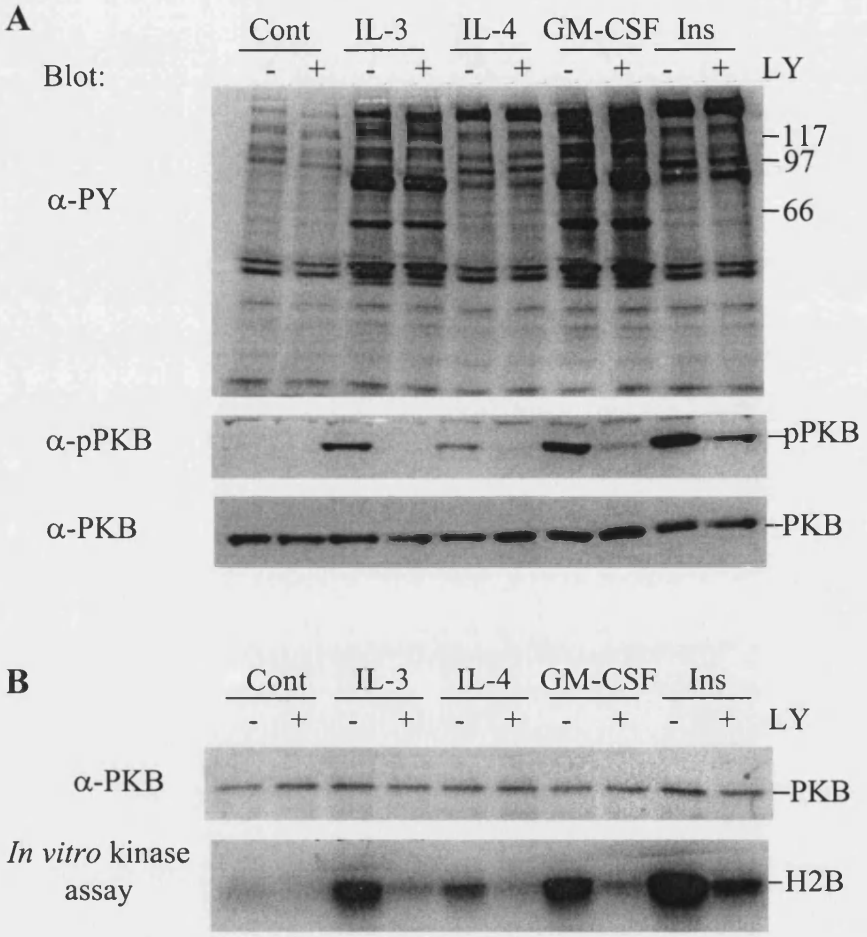
Treatment of MC9 cells with the PI3K inhibitor LY294002 for 30 minutes prior to stimulation with IL-3, IL-4, SCF, GM-CSF or insulin completely abrogated the phosphorylation of Ser^{473} induced by these cytokines (Figure 3.13A, middle panel). Similar results were observed when the effects of LY294002 pre-treatment on PKB activity was measured directly using *in vitro* kinase assays. Pre-treatment with LY294002 completely inhibited the IL-3-, IL-4- and insulin-induced activation of PKB (Figure 3.13B). Treatment of MC9 cells with LY294002 before stimulation with SCF and GM-CSF also resulted in a dramatic reduction of PKB activity, but did not reduce the activity to basal levels. These results show that in MC9 cells IL-3, IL-4, insulin, SCF and GM-CSF all activate PKB through a PI3K mediated pathway. LY294002 pre-treatment did not completely abrogate the activation of PKB in response to SCF and

GM-CSF. This could be because the PKB activation induced by these factors is too strong to be inhibited by 10μM LY294002 (as seen with insulin stimulation of FD-5 and FD-6 cells in Figures 3.11 and 3.12) or that other pathways are involved in the activation of PKB by these factors in MC9 cells.

3.13 Effect of PI3K Inhibition on PKB activation BaF/3 Cells

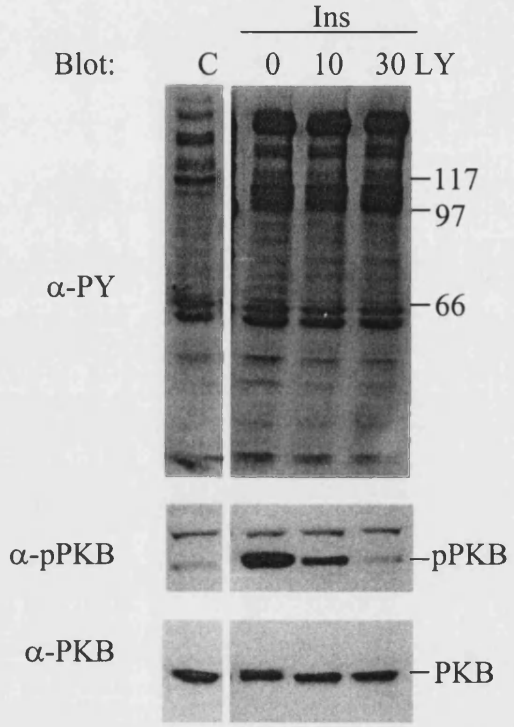
Pre-treatment of BaF/3 cells with 10μM LY294002 for 30 minutes completely abolished both the IL-3 and IL-4 induced phosphorylation of PKB on Ser^{473} (Figure 3.14A, middle panel). *In vitro* kinase assays showed that LY294002 treatment abrogated the IL-4 induced activation of PKB and reduced that induced by IL-3 (Figure 3.14B). This shows that both IL-3 and IL-4 activate PKB via a PI3K mediated pathway in BaF/3 cells. Pre-treatment with LY294002 did not effect the tyrosine phosphorylation induced by stimulation with either cytokine.

Figure 3.11
Effect of LY294002 on PKB Activation in FD-6 Cells



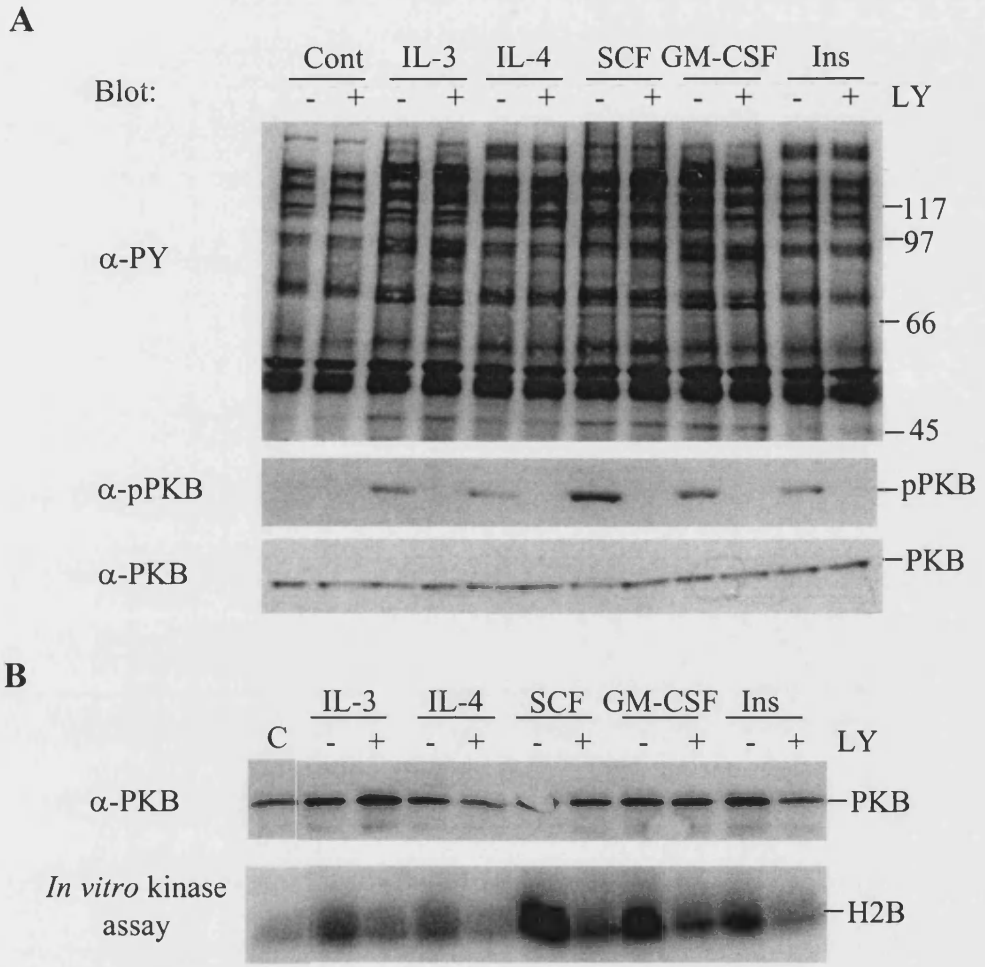
FD-6 cells were starved of serum and factor for 30 min followed by a 30 min incubation with 10μM LY294002 or carrier (DMSO) alone. Cells were stimulated with growth factor (20ng/ml IL-3 for 5 min; 20μg/ml IL-4 for 10 min; 5μg/ml GM-CSF for 5 min; 5μg/ml insulin for 2min). **(A)** Whole cell lysates were separated on duplicate gels by SDS-PAGE, transferred to nitrocellulose, and probed with α-phosphotyrosine (α-PY, upper panel) and α-phosphoSer^{473} (α-pPKB, middle panel). Blots were stripped and reprobed with α-PKB (lower panel). **(B)** Cells were lysed (10^7 cell equivalents) and immunoprecipitated with α-PKB. *In vitro* kinase assays were performed using histone2B (H2B) as the substrate and the products separated by SDS-PAGE and transferred to nitrocellulose. The membranes were cut in half, the upper immunoblotted with α-PKB (upper panel) and the lower subjected to autoradiography (lower panel). This experiment is representative of (A) 4 and (B) 3 independent experiments. The experiments shown in (A) and (B) are not performed on cell extracts from the same stimulation.

Figure 3.12
Effect of LY294002 inhibition on insulin-induced PKB acvtivation in FD-5 Cells



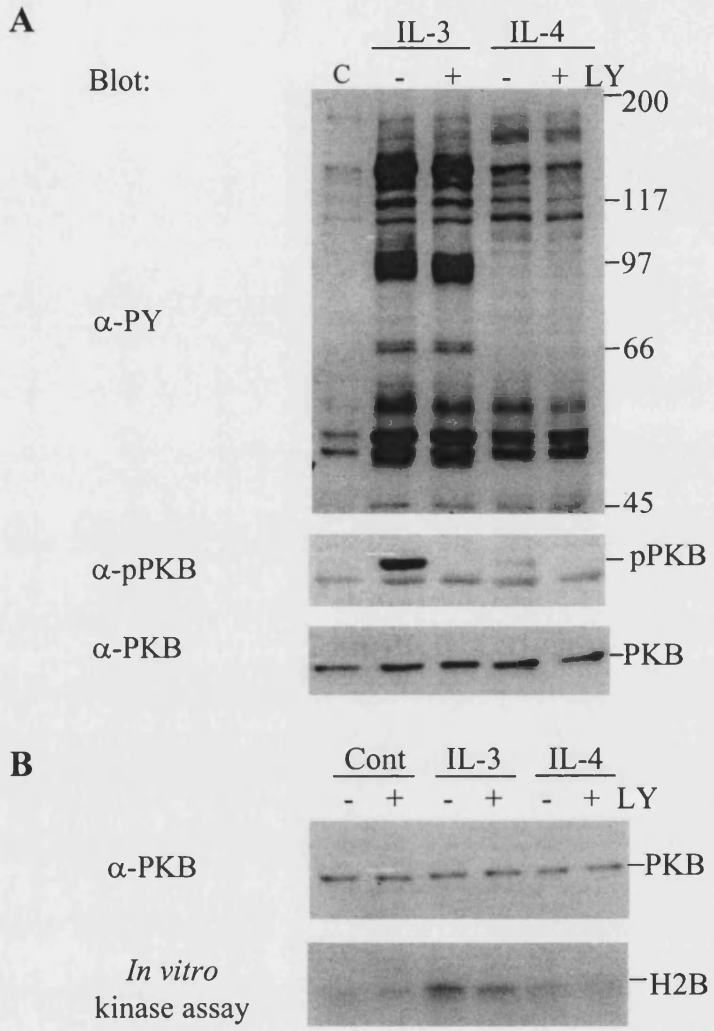
FD-5 cells were starved of factor and serum for 30 minutes followed by a 30 minute incubation with the indicated concentration of LY294002 (μM) or carrier (DMSO) alone. Cells were stimulated with 5μg/ml of insulin for 2 minutes and whole cell extracts were separated on on duplicate gels by SDS-PAGE and transferred to nitrocellulose. Blots were immuno-blotted with α-phosphotyrosine (α-PY, upper panel) and α-phosphoSer^{473} (α-pPKB, middle panel). Blots were stripped and reprobed with α-PKB (lower panel). This experiment is representative of 2 independent experiments. The experiments shown in (A) and (B) are not performed on cell extracts from the same stimulation.

Figure 3.13
Effect of LY294002 on PKB Activation in MC9 Cells



MC9 cells were starved of serum and factor for 30 min followed by a 30 min incubation with 10μM LY294002 or carrier (DMSO) alone. Cells were stimulated with growth factor (20ng/ml IL-3 for 5 min; 20μg/ml IL-4 for 10 min; 50ng/ml SCF for 5 min; 5μg/ml GM-CSF for 5 min; 5μg/ml insulin for 2 min). **(A)** Whole cell lysates were separated on duplicate gels by SDS-PAGE and transferred to nitrocellulose, and probed with α-phosphotyrosine (α-PY, upper panel) and α-phosphoSer^{473} (α-pPKB, middle panel). Blots were stripped and reprobed with α-PKB (lower panel). **(B)** Cells were lysed (10^7 cell equivalents) and immunoprecipitated with α-PKB. *In vitro* kinase assays were performed using histone2B (H2B) as the substrate and the products separated by SDS-PAGE and transferred to nitrocellulose. The membranes were cut in half, the upper immunoblotted with α-PKB (upper panel) and the lower subjected to autoradiography (lower panel). This experiment is representative of (A) 3 and (B) 2 independent experiments. The experiments shown in (A) and (B) are not performed on cell extracts from the same stimulation.

Figure 3.14
Effect of LY294002 on PKB Activation in BaF/3 Cells



BaF/3 cells were starved of serum and factor for 30 min followed by a 30 min incubation with 10μM LY294002 or carrier (DMSO) alone. Cells were stimulated with growth factor (20ng/ml IL-3 for 5 min; 20μg/ml IL-4 for 10 min). **(A)** Whole cell lysates were separated on duplicate gels by SDS-PAGE and transferred to nitrocellulose, and probed with α-phosphotyrosine (α-PY, upper panel) and α-phosphoSer^{473} (α-pPKB, middle panel). Blots were stripped and reprobed with α-PKB (lower panel). **(B)** Cells were lysed (10^7 cell equivalents) and immunoprecipitated with α-PKB. *In vitro* kinase assays were performed using histone2B (H2B) as the substrate and the products separated by SDS-PAGE and transferred to nitrocellulose. The membranes were cut in half, the upper immunoblotted with α-PKB (upper panel) and the lower subjected to autoradiography (lower panel). This experiment is representative of (A) 2 and (B) 3 independent experiments. The experiments shown in (A) and (B) are not performed on cell extracts from the same stimulation.

3.14 Comparison of the levels of PKB phosphorylation

Both PI3K and PKB have been implicated in the growth and survival of haemopoietic cells. The experiments above show that all the growth factors examined are able to induce the activation of PKB through PI3K in the responsive haemopoietic cell lines used. However, not all the growth factors examined are able to promote the long-term survival and proliferation of these cells. Therefore, the relative levels of PKB activation induced by each factor were compared to determine whether a correlation exists between the level of PKB activation and the functional responses of survival and proliferation. In addition to examining the effect of PI3K inhibition on PKB activity Figures 3.11-3.14 were used to compare the relative levels of PKB activity induced by each growth factor. Cells were stimulated for the length of time and dose known to induce maximum PKB activation/phosphorylation and both Ser^{473} phosphorylation and PKB activity examined. In MC/9 cells IL-3, IL-4, GM-CSF and insulin all activated PKB to a similar level while SCF induced a much greater level of activation (Figure 3.12). In FD-6 cells the insulin-induced activation was far greater than that induced by the other cytokines and IL-4 induced very little activation (Figure 3.11). Both IL-3 and IL-4 induced the activation of PKB in BaF/3 cells, again IL-4 to a very low level (Figure 3.14).

After autoradiography of the *in vitro* kinase assays, the histone2B bands were excised, counted in a scintillation counter and the counts used to determine the fold-increase in PKB activity following factor treatment. These are summarized in Table 3.1. The fold-activation of PKB by each cytokine varied with each experiment but the relative level of activation induced by each growth factor remained constant. For example, the insulin-induced activation of PKB in FD-6 cells was consistently much greater than the activation induced by IL-3, IL-4 and GM-CSF. The trends observed here are similar to those seen when the levels of Ser^{473} phosphorylation are compared.

Table 3.1

Fold-Activation of PKB in response to cytokine-stimulation

	Growth Factor					
		IL-3	**IL-4**	**SCF**	**GM-CSF**	**Insulin**
Cell type	**BaF/3**	2 n = 2	1.5 n = 2	ND	ND	ND
	FD-6	2.5-8 n = 6	2 n = 7	ND	2.5-5 n = 2	3-10 n = 3
	MC9	1.5-2 n = 4	1.5-2 n = 5	2.5-4 n = 4	1.5 n = 2	1.5-2.5 n = 2

In vitro kinase assays were performed as described in Figures 3.1-3.6 and 3.11, 3.13-3.14. After autoradiography the histone2B bands were excised from the blot, their radioactivity counted on a scintillation counter and the maximum fold-activation of PKB by each cytokine was determined. The values above show the range of fold-activation induced by each growth factor. ND = not determined.

3.15 Cytokine induced phosphorylation of BAD

Having established that IL-3, IL-4, SCF, insulin and GM-CSF activate PKB and that the level and kinetics of activation varies with each cytokine and cell line, it was of interest to look at the effects of these differences on substrates of PKB. At the time of this work Bad, a pro-apoptotic member of the Bcl-2 family, was reported to be phosphorylated on two sites, Ser^{112} and Ser^{136}, but subsequently a third phosphorylation site, Ser^{155}, has been identified (Zhou *et al.*, 2000; Lizcano *et al.*, 2000). The Ser^{136} site is phosphorylated by PKB in response to IL-3 stimulation and it had been proposed that this accounts for the survival signal generated by IL-3 and PKB (del Peso *et al.*, 1997; Zha *et al.*, 1996).

3.16 Hyper-Phosphorylation of Bad in MC9 Cells

Phosphorylation of Bad causes a shift in its migration through SDS-PAGE and this can be used to determine the phosphorylation status of Bad (Zha *et al.*, 1996). Bad was immunoprecipitated from cell extracts prior to SDS-PAGE through 12.5% low bis (118:1) gels and immunoblotted with anti-Bad. As previously reported (Scheid and Duronio, 1998) in MC/9 cells IL-4 and insulin induced no change in the migration of Bad indicating that they are unable to induce the hyper-phosphorylation of Bad (Figure 3.15A). IL-3, SCF and GM-CSF, however, induced the complete conversion of Bad to its slower migrating, hyper-phosphorylated form. In unstimulated MC9 cells both the slower and faster migrating forms of Bad were always detected reflecting a high basal level of phosphorylation, the reasons for which are not clear.

3.17 Hyper-Phosphorylation of Bad in FD-6 Cells

In FD-6 cells (Figure 3.15B, upper panel) IL-3 induced a complete conversion of Bad to its slower migrating, hyper-phosphorylated form and IL-4 was able to induce a partial but consistently observed conversion. Interestingly, insulin, while being the strongest inducer of PKB activation in FD-6 cells, induced a very low level of Bad hyper-phosphorylation. Pre-treatment with LY294002 decreased the mobility shift in all cases highlighting the requirement for PI3K.

PKB is thought to phosphorylate Bad on Ser^{136}, therefore, the phosphorylation of this site was examined using antibodies specific for phospho-Ser^{136}. Phospho-Ser^{136} antibodies from both NEB and UBI were tried, however, neither appeared to be specific to Bad (data not shown). Therefore, to further clarify the phosphorylation of Bad particularly in response to IL-4 and insulin this blot was stripped and reprobed with an

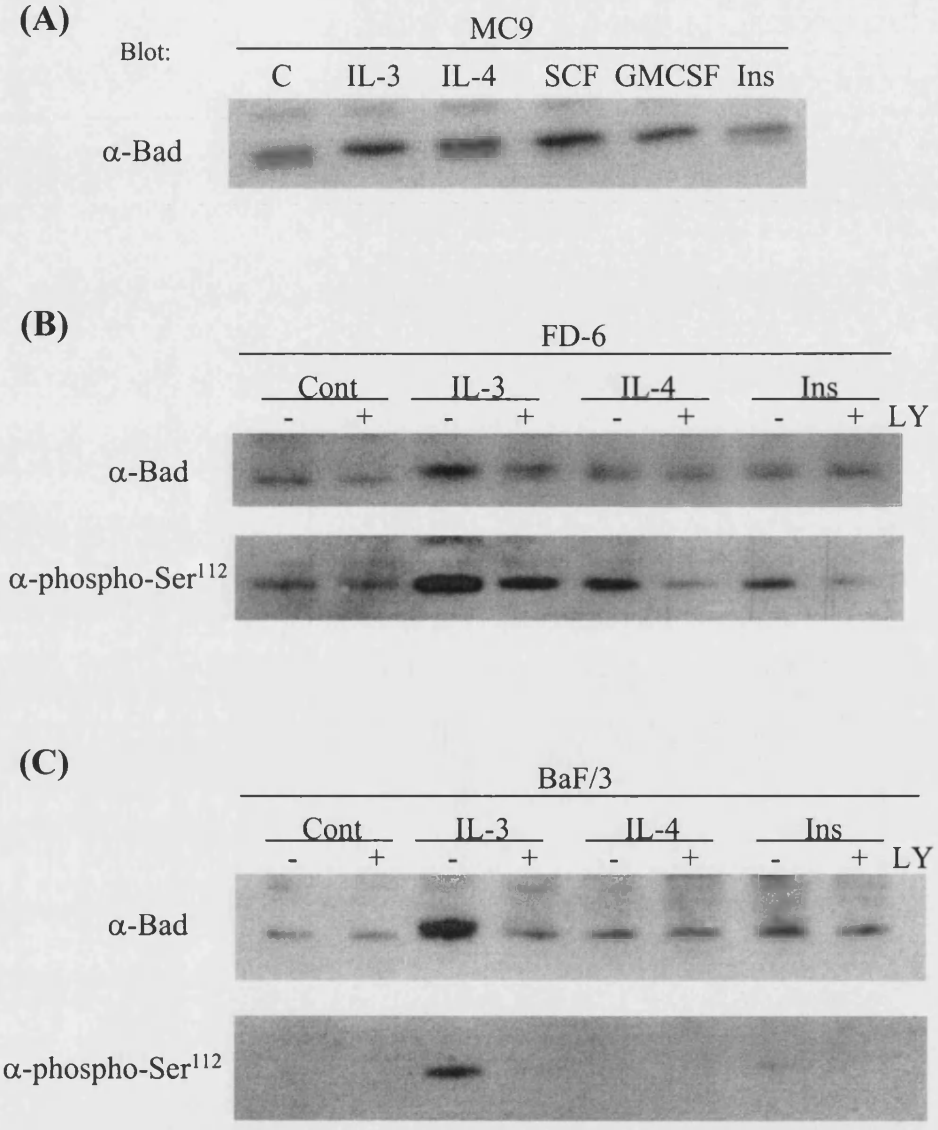
antibody reputed to be specific for phospho-Ser^{112} (Figure 3.15B lower panel). This result shows that IL-4 and insulin induce a low level of Ser^{112} phosphorylation in FD-6 cells and confirms the shift in Bad migration observed when cells are stimulated with these factors.

3.18 Hyper-Phosphorylation of Bad in BaF/3 Cells

In BaF/3 cells (Figure 3.15C, upper panel) IL-4 was unable to convert Bad to its slower migrating, phosphorylated form, insulin stimulation induced little if any change in migration, while IL-3 was a potent inducer of Bad phosphorylation. As seen in the FD-6 cell line pre-treatment with LY294002 decreased the mobility shift again highlighting the requirement for PI3K. This blot was stripped and reprobed with phospho-Ser^{112} (Figure 3.15C, lower panel). Consistent with the mobility shift data, IL-3 induced significant Ser^{112} phosphorylation and insulin induced a low level of Ser^{112} phosphorylation in BaF/3 cells. No Ser^{112} phosphorylation was observed in IL-4 stimulated BaF/3 cells.

Taken together, these results do not show a correlation between the level of PKB activation and the ability to induce Bad hyperphosphorylation as judged by a shift in mobility. Insulin is a potent activator of PKB in FD-6 cells yet barely induces the hyper-phosphorylation of Bad. Conversely, IL-4 is a relatively poor activator of PKB in this cell line yet is able to induce Bad phosphorylation. In MC/9 cells both insulin and IL-4 are able to activate PKB to levels similar to those of IL-3 and GM-CSF, yet while the latter two growth factors can induce Bad phosphorylation insulin and IL-4 cannot. Yet in BaF/3 cells there appears to be a correlation between PKB activation and Bad hyper-phosphorylation. IL-3 which is a relatively potent PKB activator, can induce Bad hyper-phosphorylation, while IL-4, which induces relatively little PKB activation is unable to phosphorylate Bad.

Figure 3.15
Cytokine induced phosphorylation of Bad



(A) MC-9 cells were starved of factor and serum for 1 hour, or **(B)** FD-6, and **(C)** BaF/3 cells were starved of factor and serum for 30 minutes followed by a 30 minute incubation with 10μM LY294002 or carrier (DMSO) alone. Cells ($2x10^7$) were stimulated at the dose and time previously shown to induce maximal PKB activation (see legend to Figures 3.11, 3.13 and 3.14) and immunoprecipitated with α-Bad, separated by SDS-PAGE and transfered to nitrocellulose. Blots were immunoblotted with α-Bad (upper panels), and **(B)** and **(C)** stripped and reprobed with α-phosphoSer^{112} (lower panels). These experiments are representative of (A) 3, (B) 5, and (C) 2 independent experiments.

3.19 Cytokine induced Survival and Proliferation

The previous results indicate that cytokine-induced activation of PKB does not necessarily result in the hyper-phosphorylation of Bad. As both of these events had previously been thought to be important for cellular survival (Datta *et al.*, 1997), it was of interest to investigate which cytokines could support the survival and proliferation of BaF/3 and FD-6 cell lines. These factor-dependent cell lines are useful models for studying cell survival and proliferation because they will cease to proliferate and undergo apoptosis in the absence of a survival signal.

3.20 Survival and proliferation of FD-6 Cells

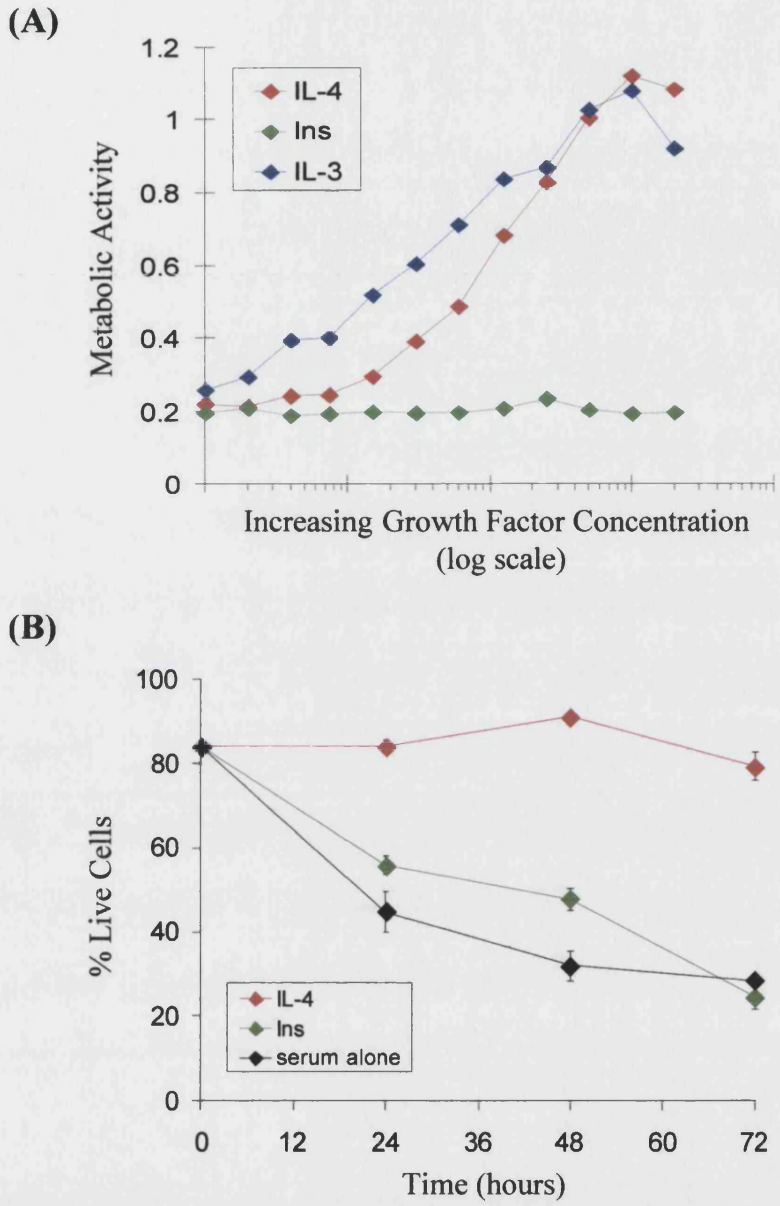
XTT dye reduction assays were used to measure the proliferation/survival of FD-6 cells in response to IL-3, IL-4 and insulin. These assays measure the bio-reduction of XTT to its soluble, coloured formazan product, giving an indication of cellular metabolic activity and hence proliferation and survival of cells. In 72 hour XTT dose response assays both IL-3 and IL-4 supported the proliferation of FD-6 cells (Figure 3.16A), however, these cells did not proliferate in response to insulin concentrations of 0.1pg/ml to 1μg/ml.

To investigate the role that insulin and IL-4 can play in the survival of FD-6 cells viability assays were performed. FD-6 cells were grown in IL-4, insulin or serum alone and the percentage of viable cells were determined by dye exclusion assays. Live cells were distinguished from dead ones by their exclusion of trypan blue and the percentage of each determined. Cells grown in IL-4 maintained a viability of approximately 85% (Figure 3.16B) over 72 hours. Insulin appeared to give some protection from apoptosis in the short term, the viability of insulin treated cells was approximately 10% greater than that of cells in serum alone at 24 and 48 hours but by 72 hours no difference was seen. Therefore insulin, despite being a potent activator of PKB in FD-6 cells, is unable to provide a signal for these cells to survive and proliferate.

3.21 Survival of BaF/3 Cells

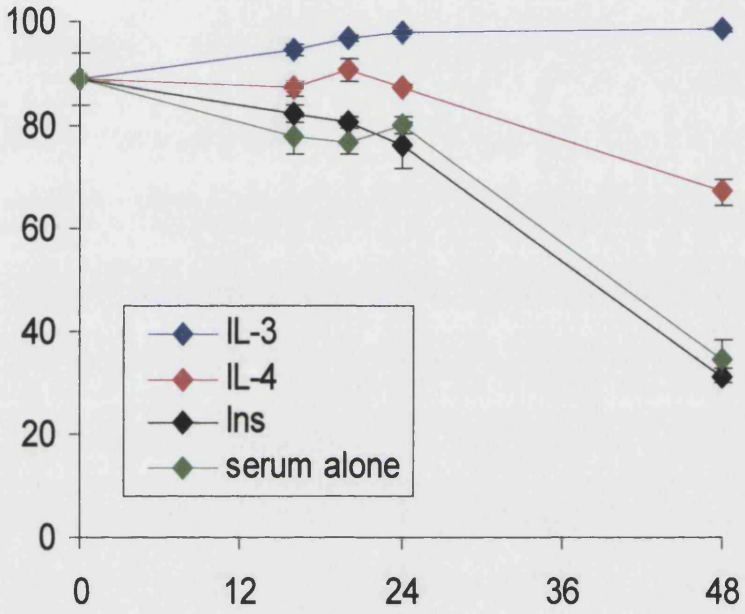
Viability assays were performed to investigate the ability of IL-3, IL-4 and insulin to promote the survival of BaF/3 cells. Cells grown in IL-3 maintained a viability of greater than 90% over 48 hours (Figure 3.17). IL-4, despite inducing a very low level of PKB activation and not promoting the dual phosphorylation of Bad, appeared to afford some protection from apoptosis. However, insulin was unable to sustain viability above the levels of serum alone.

Figure 3.16
Viability and Proliferation of FD-6 cells in response to cytokines



(A) FD-6 cells were washed free of factor and set up with growth factor (at a starting concentration of IL-3, 2ng/ml; IL-4 10ng/ml: insulin 1μg/ml) or serum alone with serial 1:2 dilutions across a 96 well plate as described in materials and methods. After 72 hours the assays were developed and the mean values with standard error are plotted. **(B)** FD-6 cells were washed free of factor and set up with growth factor at a concentration of IL-3, 2ng/ml; IL-4 10ng/ml: insulin 1μg/ml or serum alone. The percentage of viable cells was determined by the exclusion of trypan blue. Values are the average of 4 counts and are plotted with standard error.

Figure 3.17
Viability of BaF/3 Cells in Response to Cytokines



BaF/3 cells were washed free of IL-3 and serum and seeded at 10^5/ml in 2ng/ml IL-3, 10ng/ml IL-4, 1μg/ml insulin or 10% v/v serum only. The percentage of viable cells was determined at the specified times by their exclusion of trypan blue. Values are the average of 4 counts and are plotted with standard error. This experiment is representative of 3 independent experiments.

Table 3.2

Summary of Cytokine Responses

Cell type	Cytokine	PKB activation	Bad Phosphorylation	Survival	Proliferation
Baf/3	IL-3	Yes	Yes	Yes	Yes
	IL-4	Yes	No	Limited	No
FD-6	IL-3	Yes	Yes	Yes	Yes
	IL-4	Yes	Yes	Yes	Yes
	Insulin	Yes	Yes	No	No
MC-9	IL-3	Yes	Yes	Yes	Yes
	IL-4	Yes	No	ND	ND
	SCF	Yes	Yes	ND	ND
	Insulin	Yes	No	ND	ND
	GM-CSF	Yes	Yes	ND	ND

The effects of cytokine simulation of each cell line on PKB activation, Bad phosphorylation and the correlation of these events with survival and proliferation are summarised. This table highlights the finding that a definite and consistent correlation between PKB activation, Bad phosphorylation and cell survival and proliferation does not exist.

3.22 Discussion

PI3K and its downstream effector PKB have been implicated in the generation of survival and proliferative signals in a number of cell types and in response to a variety of stimuli (Dudek *et al.*, 1997; Kauffman-Zeh *et al.*, 1997; Kennedy *et al.*, 1997; Khwaja *et al.*, 1997; Songyang *et al.*, 1997). However, the question of whether PKB activation is absolutely required or necessary for the generation of a survival/proliferative signal had not been investigated. This issue was addressed by investigating cytokine-induced activation of PKB and whether this is sufficient for the continued growth and survival of cells. The results presented here show that an absolute correlation between PKB activation, cell survival and proliferation does not exist.

Cytokines are known to act as growth and survival factors for haemopoeitic cells (Metcalf, 1989). All the cytokines examined activated PKB in a PI3K-dependent manner, as this activation was sensitive to inhibition by LY294002 (Figures 3.11-3.14). However, not all the cytokines were able to induce a mobility shift in Bad due to hyper-phosphorylation, and not all cytokines were sufficient to provide a survival and/or proliferative signal to the cells. In FD-6 cells, insulin was a potent activator of PKB, increasing the activity by up to 10-fold (Table 3.1). However, insulin was limited in its ability to promote the survival of these cells. In 72hr XTT assays insulin was unable to promote growth or survival of FD-6 cells (Figure 3.16A), and in viability assays, while insulin perhaps gave partial protection from apoptosis at 24 and 48hrs, by 72hrs it afforded no more protection than serum alone (Figure 3.16B). Contrasting with insulin, IL-4, which induced only 2-fold activation of PKB in this cell type (Table 3.1), was able to maintain the viability of FD-6 cells at 85% (Figure 3.16B) and induced their proliferation as measured in XTT assays (Figure 3.16A). This demonstrates that activation of PKB does not automatically lead to the generation of a survival signal.

One downstream effector of the PKB survival pathway is the pro-apoptotic member of the Bcl-2 family, Bad. Phosphorylation of Bad prevents its interaction with the anti-apoptotic family members Bcl-2 and Bcl-X_L allowing them to inhibit apoptosis. A number of serine phosphorylation sites have been identified on Bad and further investigations have identified the kinases that phsophorylate these sites: Ser^{112} is phosphorylated by MAPKAP-K1 (RSK) via the MAP kinase pathway (Bonni *et al.*, 1999), Ser^{136} is phosphorylated by PKB (Zha *et al.*, 1996) and Ser^{155} by PKA (Lizcano

et al., 2000). The separation of Bad on SDS-PAGE gels results in a doublet, the lower band consisting of both hypo- and un-phosphorylated protein and the upper of hyper-phosphorylated Bad (Zha *et al.*, 1996). However, it is unclear how many sites on Bad need to be phosphorylated in order to see a band shift. This shift in migration was used to examine the phosphorylation status of Bad in response to cytokine stimulation. In all cell types examined, IL-4 was able to activate PKB, yet this did not necessarily result in the hyper-phosphorylation of Bad. It had been previously reported that IL-4 was unable to promote the hyper-phosphorylation of Bad in MC-9 cells (Scheid and Duronio, 1998) and similar results were observed here in both MC9 (Figure 3.15A) and BaF/3 (Figure 3.15C) cells. However, this may not be a universal phenomenon as cell-type differences were discovered. In FD-6 cells a consistent, but low level of Bad hyper-phosphorylation was observed in response to IL-4 stimulation and phosphorylation of Ser^{112} could be detected (Figure 3.15B). Given that IL-4 is unable to induce the hyper-phosphorylation of Bad in MC9 and BaF/3 cells this is a surprising result. IL-4 is able to promote the long-term survival and proliferation of FD-6 cells and while it does afford limited protection from death in BaF/3 cells (Figure 3.17) it is not sufficient to promote the long-term growth and survival of either BaF/3 or MC9 cells. One interpretation of this result is a requirement for Bad hyper-phosphorylation to drive the long-term survival and proliferation of cells. This hypothesis is supported by the finding that in BaF/3 cells, which are dependent on IL-3 for their continued growth and survival, IL-3 but not IL-4 or insulin was able to induce the hyper-phosphorylation of Bad (Figure 3.15C). However, insulin also promotes a low but consistently observed level of Bad phosphorylation in FD-6 cells (Figure 3.15B), yet it is unable to promote the growth or survival of these cells (Figure 3.16).

Bad is phosphorylated by PKB on Ser^{136} (Datta *et al.*, 1997) but attempts to directly investigate the phosphorylation at this site using phospho-specific antibodies were unsuccessful. However, the cytokine-induced phosphorylation of Bad on Ser^{112} was examined in FD-6 and BaF/3 cells (Figure 3.15B and C). The kinase that phosphorylates Bad on Ser^{112} has recently been identified as MAPKAP-K1. Given that the immediate upstream regulators of MAPKAP-K1, Erk1 and 2, are not activated by IL-4 in the FD-6 parental cell line FDMAC11/4.5 (Welham *et al.*, 1994b) it is surprising that we observed phosphorylation of Ser^{112} in response to IL-4 stimulation. However, a recent study found that the NEB phospho-Ser^{112} antibody also binds, albeit to a lesser extent, to phosphorylated Ser^{136} and Ser^{155} residues (Lizcano *et al.*, 2000). Therefore

phosphorylation of these residues may account for the "Ser^{112}" phosphorylation observed in response to IL-4 stimulation of FD-6 cells.

In BaF/3 cells IL-3 was able to convert Bad to its hyper-phosphorylated form. This effect was partially inhibited by pre-treatment with LY294002 indicating that it is mediated at least in part through PI3K. In BaF/3 cells PI3K inhibition has been shown to inhibit both PKB (this study) and the MAPKAP-K1 regulators the Erk 1 and 2 (Craddock *et al.*, manuscript submitted). Thus, despite inhibition of two of the known Bad kinases, the IL-3 mediated hyper-phosphorylation is only partially blocked. It is unclear how many residues on Bad need to be phosphorylated in order to induce the shift in migration but as serine phosphorylated Bad migrates in the lower band along with un-phosphorylated protein (Zha *et al.*, 1996) it would appear that more than one residue must be phosphorylated. This suggests that either other physiological kinases responsible for phosphorylation at Ser^{112} and Ser^{136} exist or there are additional serine phosphorylation sites on Bad. Recent work has suggested that Ser^{170} is also a site of phosphorylation on Bad (V. Duronio, personal communication) and phosphorylation of this site could contribute to the shift in migration observed in the absence of PI3K activity.

Insulin was the most potent activator of PKB in FD-6 cells (Figures 3.11-3.14) and it has also been shown to activate Erk1 and Erk2 to its sister cell line FD-5 (Welham *et al.*, 1995). In contrast, IL-4 induced a relatively weak activation of PKB in FD-6 cells and is unable to activate Erk1 and Erk2 in the parental cell line FDMAC4.5/11 (Welham *et al.*, 1994b). Given this, it is surprising that insulin was not more effective than IL-4 at inducing the hyper-phosphorylation and especially the Ser^{112} phosphorylation of Bad in FD-6 cells (Figure 3.15B). The strength of PKB activation observed and the fact that the activating MAPKAP-K1 kinases, Erk1 and Erk2, are activated in response to insulin suggest that the activation of these kinases alone is not sufficient to promote the hyper-phosphorylation of Bad. Lizcano *et al.* (2000) reported that the phospho-specific Ser^{112} antibody used in these experiments also recognised phosphorylated Ser^{136} and Ser^{155} residues but with a lower affinity. Therefore it is unclear whether the insulin-induced "Ser^{112}" phosphorylation observed is due to Ser^{112}, Ser^{136} or Ser^{155} phosphorylation, although, phosphorylation at any of these serine residues lessens the affinity of Bad for Bcl-2 and Bcl-X_L (Zhou *et al.*, 2000; Zha *et al.*, 1996). It would be interesting to examine which of these sites are phosphorylated in

response to insulin and IL-4 stimulation to determine which pathways are important for the phosphorylation of Bad in these cells.

The apoptotic functions of the Bcl-2 family are thought to be regulated in part by the relative concentrations of pro- and anti-apoptotic members (Oltvai and Korsmeyer, 1994). When Bad is phosphorylated it interacts with 14-3-3 proteins (Zha *et al.*, 1996). This effectively removes Bad from the Bcl-2 family pool, thereby increasing the concentration of anti-apoptotic family members, which promote cell survival (Section 1.3.2). In FD-6 cells, IL-3 was the most efficient factor at phosphorylating Bad. IL-4 and insulin to a lesser extent were also able to induce Bad phosphorylation in this cell line. Given that the relative levels of Bcl-2 family members are thought to be the important factor governing their role in apoptosis, it is surprising that IL-4 despite inducing far less Bad hyper-phosphorylation that IL-3, is just as efficient at promoting the growth and survival of FD-6 cells. In contrast, insulin is unable to promote the growth and survival of FD-6 cells, yet the level of Bad phosphorylation induced by insulin is only marginally less than that induced by IL-4. It is difficult to determine what level of Bad phosphorylation is physiologically relevant to the survival of the cell and as the relative levels of Bcl-2 proteins seem to be important the effects of growth factor-stimulation on the expression and "activity" of the other Bcl-2 family members must be considered. As such, using gel-shifts to look at the hyper-phosphorylation of Bad can only give an indication of the general level of Bad phosphorylation. This does not automatically mean that the cell is receiving a survival signal.

A number of techniques could be used to investigate which site(s) on Bad are phosphorylated in response to cytokine stimulation and which of these are functionally relevant to cell survival. A phospho-peptide mapping technique could be used. Bad immunoprecipitates from cytokine treated and ^{32}P labelled cells would be subjected to tryptic digestion and the products resolved in two dimensions by electrophoresis followed by ascending chromatography. This technique could be used to compare which phospho-peptides are produced by each cytokine. However, the identity of the phosphorylated residues would need to be determined by a phospho-amino acid analysis or a mass spectroscopic approach.

Alternatively, a molecular approach could be taken, whereby the phosphorylation sites on Bad (ie. Ser^{112}, Ser^{136}, Ser^{155} and Ser^{170}) are mutated in turn. These Bad variants could be transfected into cells and the cytokine-induced phosphorylation investigated either by looking at the incorporation of ^{32}P, or by gel shifts. The advantage of using this system is that the effects of these Bad variants on cell survival could also be investigated.

The results presented in this chapter suggest that an absolute correlation between the PI3K mediated activation of PKB, Bad phosphorylation and cell survival do not exist. The activation of PKB did not necessarily result in Bad phosphorylation, nor did PKB activation and Bad phosphorylation necessarily result in the survival of the cells. These findings question the widely accepted dogma that the activation of PKB is necessary and sufficient for cell survival.

Chapter 4

The Role of the PI3K/PKB Pathway in Proliferation

4.1 Introduction

The results described in Chapter 3 demonstrated that all the cytokines tested activate PKB in responsive cell lines and that this activation is mediated through PI3K. The PI3K/PKB pathway has been widely implicated in the survival of many cell types (Dudek *et al.*, 1997; Kennedy *et al.*, 1997; Kauffman-Zeh *et al.*, 1997; Songyang *et al.*, 1997; Datta *et al.*, 1997). However, from the results presented in Chapter 3, it appears that there may be cell type specific differences in the requirement of PKB for the generation of a survival signal.

The fact that only a limited correlation between PKB activation, Bad phosphorylation and cellular survival was observed pointed to the possibility that these PI3K dependent events may be more important for pathways other than survival. The PI3K and PKB pathway has also been implicated in the regulation of cellular proliferation (Brennan *et al.*, 1997; Ahmed *et al.*, 1997; Craddock *et al.*, 1999). Therefore the aim of this chapter was to examine the role that PI3K plays in the proliferation of FD-6 and BaF/3 cells and to determine if PKB is involved in this pathway.

To investigate the role of PI3K in proliferation, two approaches were used to inhibit PI3K activity. Firstly, the pharmacological PI3K inhibitor LY294002 was used to block PI3K activity in both FD-6 and BaF/3 cells and secondly, the role of PI3K in the IL-3-driven proliferation of BaF/3 cells was further examined using a stable BaF/3 cell line that inducibly expresses a dominant negative PI3K (Δp85) (Craddock *et al.*, 1999). In order to identify the role, if any, that PKB plays in the IL-3-induced proliferation of BaF/3 cells, the PKB signalling pathway was reconstituted in these Δp85 cells by the expression of a constitutively active PKB variant (gagPKB). The tetracycline-regulated gene expression system was used to express both Δp85 and gagPKB because this system allows tight control over the expression of exogenous protein. Given the putative roles of PI3K and PKB in survival and proliferation, this was considered vital. These cell lines provide elegant models in which to study the effects of PI3K and PKB in IL-3 driven proliferation.

4.2 The Effects of PI3K inhibition on cytokine induced proliferation

BaF/3 cells are dependent on IL-3 and FD-6 cells on either IL-3 or IL-4 for their continued growth and survival. In the previous chapter it was demonstrated that these factors activate PKB through PI3K in BaF/3 and FD-6 cells. PI3K and PKB have been implicated in both proliferation and survival in a number of cell types (Brennan *et al.*, 1997; Ahmed *et al.*, 1997). However, the activation of PKB by specific cytokines in BaF/3 and FD-6 cells did not correlate with the long-term survival of these cell lines in all cases. To determine whether the PI3K/PKB pathway is involved in proliferation, the effect of PI3K inhibition on the proliferation of BaF/3 and FD-6 cells was examined. To measure proliferation, XTT dye-reduction assays were used. These assays measure the bio-reduction of XTT to its soluble coloured formazan product and are in effect a measure of the metabolic activity of the cell. The results obtained from XTT assays were previously found to closely mimic those obtained when DNA synthesis was examined by measuring ^{3}H-thymidine incorporation (Craddock *et al.*, 1999). Therefore, XTT assays can be used to measure the growth and proliferation of a population of cells.

4.3 Effect of LY294002 on the Proliferation of FD-6 Cells

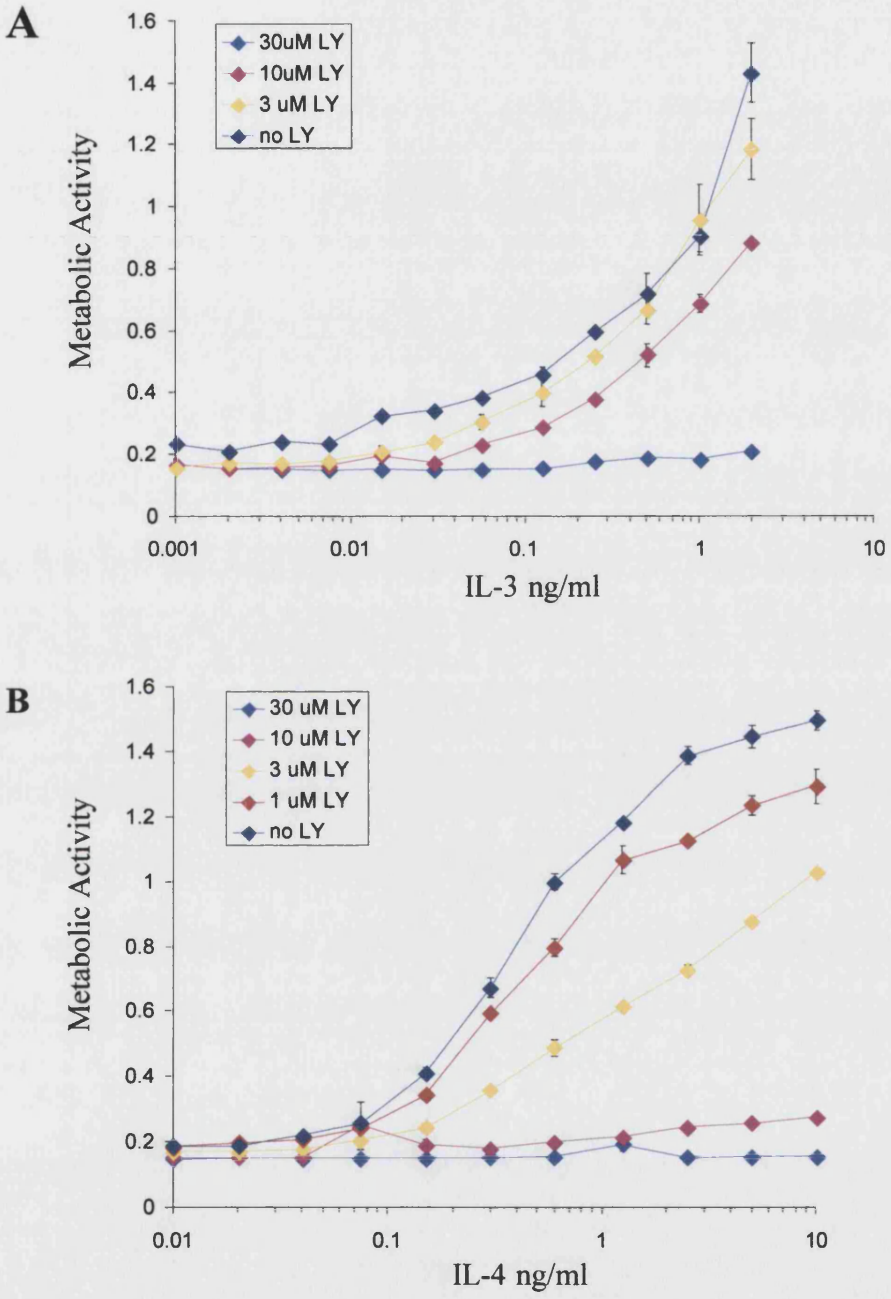
PI3K was inhibited in FD-6 cells by concentrations of LY294002 and XTT assays were used to measure the IL-3 and IL-4-driven proliferation. Both the IL-3- (Figure 4.1A) and IL-4- (Figure 4.1B) induced proliferation were inhibited in a dose-dependent manner by LY294002. IL-4 induced proliferation was more sensitive to PI3K inhibition than that of IL-3; LY294002 concentrations of less than 3μM did not significantly effect proliferation in response to IL-3, while IL-4-driven proliferation was inhibited by concentrations of LY294002 as low a 1μM. These data demonstrate that PI3K is important for the IL-3- and IL-4-induced proliferation of FD-6 cells.

4.4 Effect of LY294002 on the Proliferation of BaF/3 Cells

The effect of PI3K inhibition by LY294002 on the IL-3-induced proliferation of BaF/3 cells was measured using XTT assays. Figure 4.2 shows that the IL-3-induced proliferation of BaF/3 cells was inhibited in a dose-dependent manner by LY294002. This indicates that PI3K is needed for the IL-3-driven proliferation of BaF/3 cells.

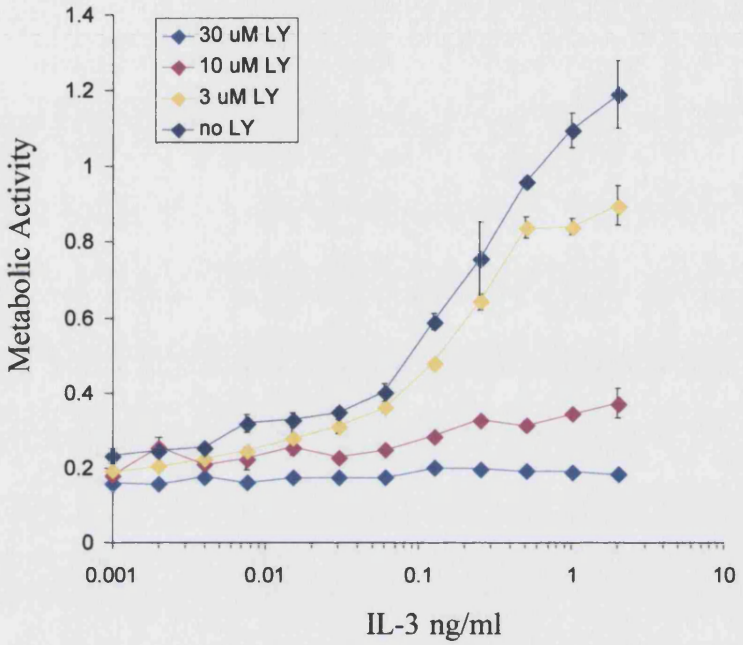
These data (Figures 4.1 and 4.2) highlight the requirement of PI3K for the IL-3 and IL-4-induced proliferative pathways.

Figure 4.1
Effect of LY294002 on Proliferation of FD6 Cells



BaF/3 cells were washed free of factor and set up in triplicate with growth factor (at a starting concentration of 2ng/ml IL-3 **(A)** or 10ng/ml IL-4 **(B)** with serial 1:2 dilutions across a 96 well plate), or serum alone as described in material and methods. The appropriate dose of LY294002 was added and after 72 hours the assays were developed. The mean values (n=3) with standard deviation are plotted.

Figure 4.2
Effect of LY294002 on Proliferation of BaF/3 Cells



BaF/3 cells were washed free of factor and set up in triplicate with IL-3 (at a starting concentration of 2ng/ml with serial 1:2 dilutions across a 96 well plate), or serum alone as described in material and methods. The appropriate dose of LY294002 was added and after 72 hours the assays were developed. The mean values (n=3) with standard deviation are plotted.

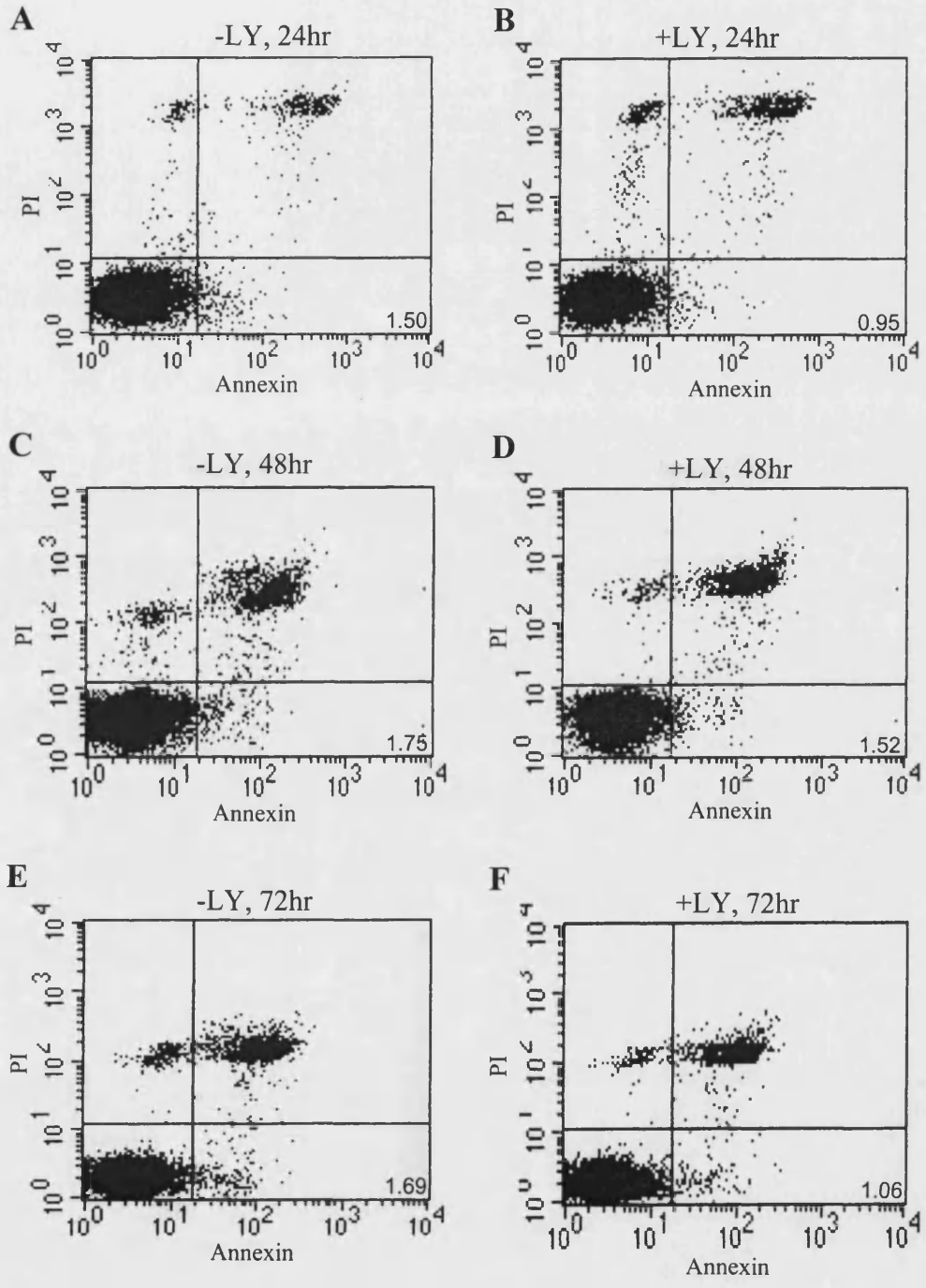
4.5 Effect of PI3K inhibition on Apoptosis in BaF/3 Cells

XTT assays measure the net metabolic activity of a population of cells. A high reading can be obtained either from a population of cells that are metabolically active, a large population of cells or a combination of the two. Therefore, while XTT assays primarily measure the proliferation of a population of cells they can also be influenced by the rate of apoptosis in the given population. To confirm that the decrease in metabolic activity observed after PI3K inhibition by LY294002 is due to a decrease in proliferation rather than an increase in apoptosis, the effects of PI3K inhibition on apoptosis were examined. FACS analysis of cells stained with propidium iodide and annexin V was used to compare the number of cells undergoing apoptosis in the presence and absence of LY294002. Figure 4.3 compares the dot plots of BaF/3 cells at 24, 48 and 72 hours after PI3K inhibition by LY294002 (+ LY) with control cells (-LY). Cells undergoing apoptosis are stained by annexinV only and these are present in the lower right quadrant. PI3K inhibition with LY294002 did not increase the numbers of cell undergoing apoptosis. These results are consistent with the view that the decrease in metabolic activity observed in the presence of LY294002 is due to a decrease in proliferation and not an increase in apoptosis.

4.6 Dominant-Negative PI3K (Δp85)

In the above experiments PI3K activity was inhibited using LY294002, which is potent against most classes of PI3K. To investigate more specifically the role of class I_A PI3K in PKB activation and proliferation, BaF/3 cells that express a dominant negative class I_A PI3K (Δp85) were used (Craddock *et al.*, 1999). Class I_A PI3Ks consist of a regulatory p85 subunit and catalytic p110 subunit (see Section 1.5.4). Δp85 lacks amino acid residues 479-513, which is the region of p85 that interacts with the p110α catalytic subunit. This prevents the interaction of Δp85 with p110. Δp85 acts as a dominant negative by competing with endogenous p85 subunits for binding through its SH2 domains to phosphorylated YXXM motifs on effector/receptor molecules. As Δp85 does not bind p110, p110 is excluded from this activating complex and this prevents it from undergoing the conformational change, which results in the activation of its catalytic activity.

Figure 4.3
Effect of LY294002 on Apoptosis



BaF/3 cells were set up at 10^4/ml either in the presence (+LY) or absencce (-LY) of 10μM LY294004. At the specified times, cells were washed and stained with annexinV and propidium iodide. Cells were analysed by flow cytometry and 10 000 events were recorded.

4.7 The Tetracycline-Regulated Expression System

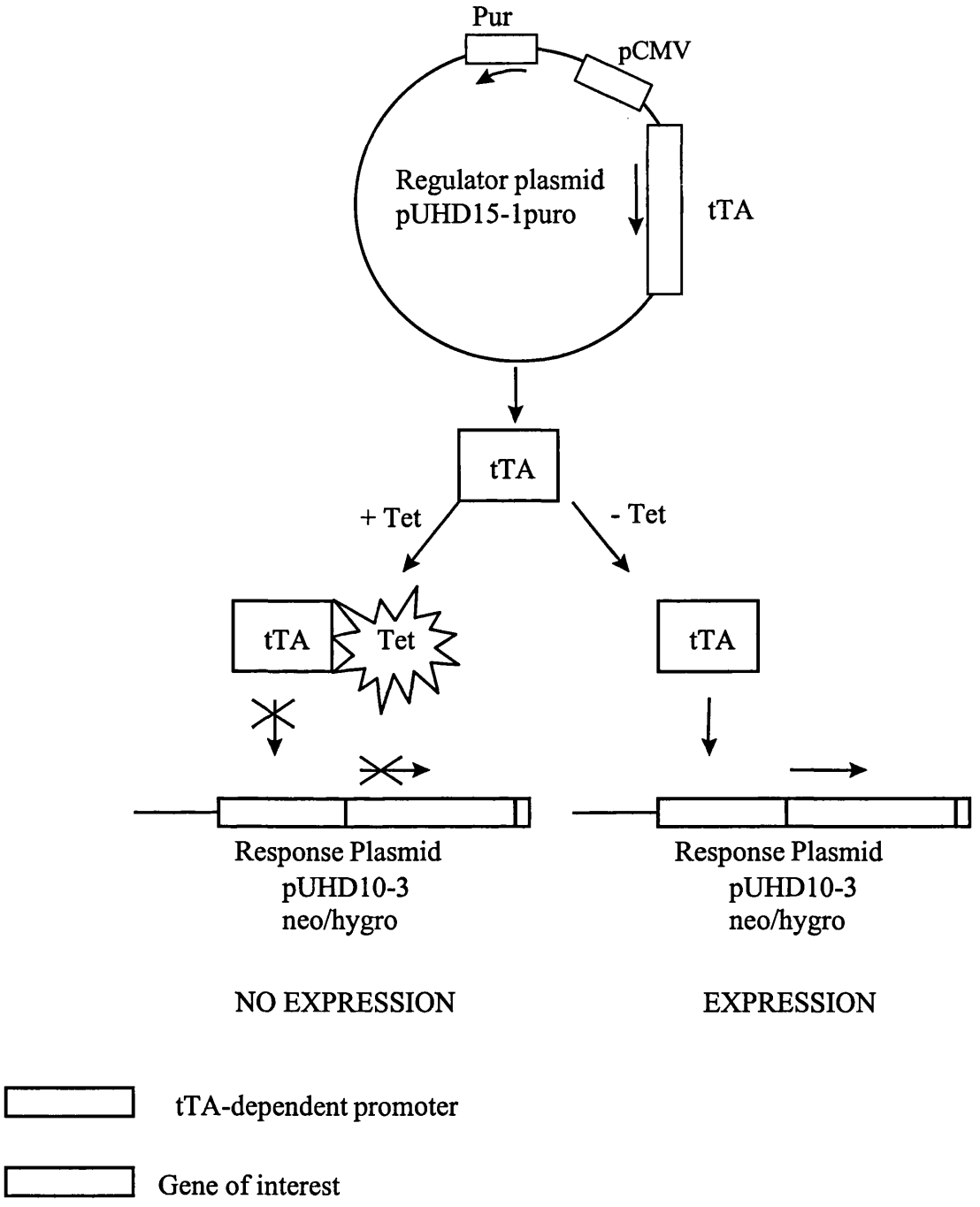
As the PI3K/PKB pathway has been implicated in the growth and survival of many cell types it was thought that the expression of dominant negative forms of these kinases could be lethal to the cell. Therefore, tight control over the expression of these proteins was considered vital. The tetracycline-regulated expression system allows the levels of expressed protein to be regulated by the addition or removal tetracycline (Gossen and Bujard, 1992). It is a two-vector system based on the repression rather than induction of expression. Systems that are based on repression remove the concern that any effects that are observed may be non-specific and caused by the addition of the drug that is used to induce gene expression (for example dexamethasone).

The two vectors used in the tetracycline-regulated gene expression system are the regulator plasmid (pUHD15-1puro) and the response plasmid (pUHD10-3neo or hygro). The regulator plasmid encodes the tetracycline-sensitive transactivator, tTA. tTA is under the control of the CMV promoter/enhancer, and followed by the SV40 polyA signal. It contains a puromycin resistance marker for the selection of transfected cells (Mui *et al.*, 1996). The response plasmid has a tTA-dependent promoter upstream of the multiple cloning site (MCS) where the gene of interest is inserted. Two forms of this plasmid were used in this study, one with a neomycin selective marker and one with a hygromycin selective marker. The two selection markers make it possible to create cell lines expressing two different mutant proteins. In this instance, it was used to express both Δp85 and gagPKB concurrently.

In this system, the presence of tetracycline represses the activity of the tetracycline-sensitive transactivator (tTA) and prevents it binding to the tTA-dependent promoter. This prevents the gene of interest on the response plasmid from being expressed. Removal of tetracycline allows tTA to bind the promoter in the response plasmid and drive expression of the cDNA of interest (Figure 4.4).

Figure 4.4

Two-vector tetracycline controlled regulatory gene expression system



A tetracycline sensitive transactivator, tTA, is encoded by the regulator plasmid pUHD15-1puro. In the presence of tetracycline, the tTA is inactive and so is prevented from binding to the tTA-dependent promoter of the response plasmid, pUHD10-3neo/hygro, and the gene of interest is not expressed. Upon removal of tetracycline, tTA can bind to the promoter, resulting in expression of the gene.

4.8 Effect of dominant-negative PI3K (Δp85) Expression on PKB phosphorylation

In BaF/3 cells, the IL-3-induced activation of PKB was significantly reduced by treatment with the PI3K inhibitor LY294002 (Figure 3.14). LY294002 is potent against all three classes of PI3K. Therefore, to specifically examine the role of class I_A PI3K in PKB activation the BaF/3(Δp85) cell line that was discussed in section 4.6 was used. In this cell line the dominant negative class I_A PI3K (Δp85) is inducibly expressed under the control of tetracycline (Craddock *et al.*, 1999). The BaF/3(Δp85) clone, 1D8 was used in this work. Antibodies specific to both PKB-Thr^{308} and PKB-Ser^{473} were used to examine the activation of PKB. Phosphorylation of both these sites is required for the optimal activation of PKB (Alessi *et al.*, 1996) and previous work showed that phosphorylation of Ser^{473} is a good indicator of the activity of PKB (Figures 3.1-3.14).

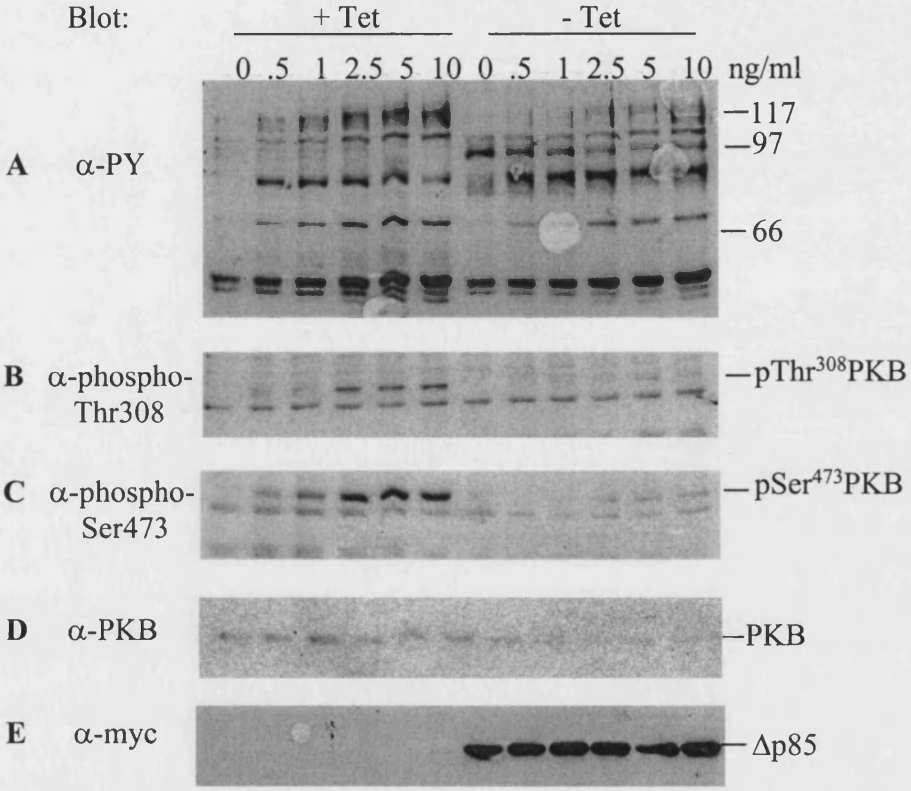
4.9 Dose response analysis of PKB Phosphorylation

In the presence of tetracycline (and therefore absence of Δp85) IL-3 induced the phosphorylation of PKB at both Ser^{473} and Thr^{308} in a dose responsive manner (Figure 4.5). As with un-transfected BaF/3 cells, maximal phosphorylation was observed at 10ng/ml IL-3. Expression of Δp85 dramatically reduced the IL-3-induced phosphorylation of both PKB-Ser^{473} and PKB-Thr^{308}, indicating that classI_A PI3Ks are involved in the IL-3-induced activation of PKB in BaF/3 cells. Although this experiment was only performed once personally, it reproduces work previously published by the laboratory (Craddock *et al.*, 1999).

4.10 Kinetic analysis of PKB Phosphorylation

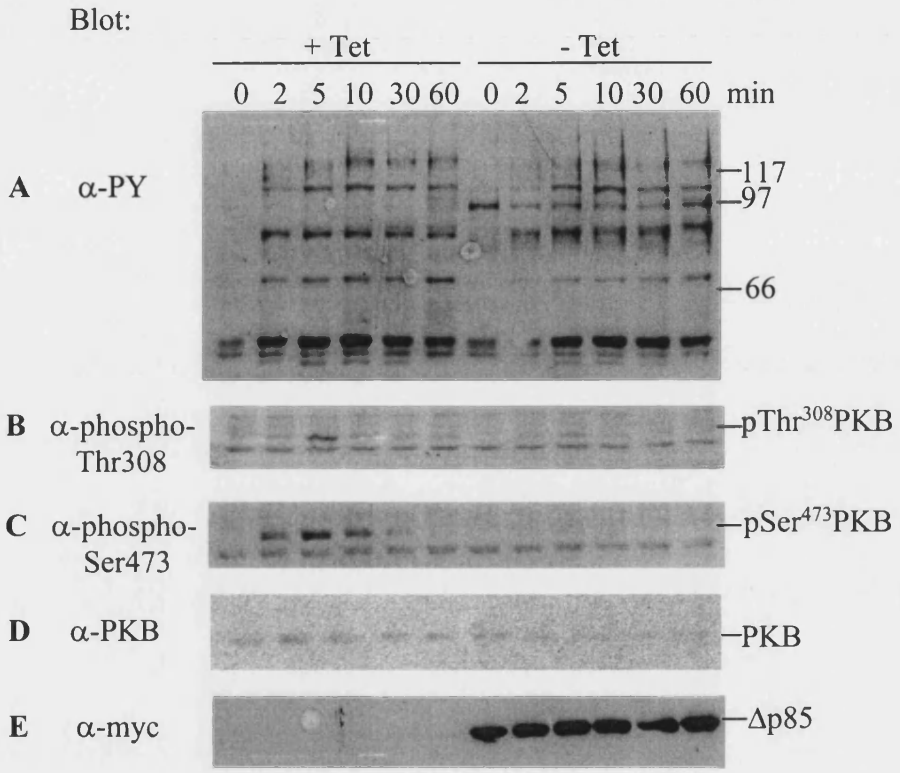
As expected from work on un-transformed BaF/3 cells (Figure 3.6), in the presence of tetracycline IL-3 phosphorylated PKB at both Ser^{473} and Thr^{308} with maximum phosphorylation observed at 5 minutes (Figure 4.6). Withdrawal of tetracycline and the subsequent expression of Δp85 significantly reduced the IL-3-induced phosphorylation of both sites. Although this experiment was only performed once personally, it reproduces work previously published by the laboratory (Craddock *et al.*, 1999).

Figure 4.5
Effect of Δp85 on PKB phosphorylation: Dose response analysis



Cells were set up at 10^5/ml in the absence (- Tet) or presence (+ Tet) of tetracycline for 24hr prior to starving of IL-3 and serum for 4hrs. Cells were stimulated for 5 minutes with the indicated concentration of IL-3. Whole cell lysates ($4x10^5$ cell equivalents) were separated be SDS PAGE on duplicate gels and immunblotted with **(C)** α-phospho-Ser^{473} or **(B)** α-phospho-Thr^{308}. Blots were stripped and reprobed with **(D)** α-PKB, **(A)** α-phospho-tyrosine and **(E)** α-myc-tag. This experiment is a repeat of work published (Craddock *et al.* 1999).

Figure 4.6
Effect of Δp85 on PKB phosphorylation: Kinetic analysis



Cells were set up at 10^5/ml in the absence (- Tet) or presence (+ Tet) of tetracycline for 24hr prior to starving of IL-3 and serum for 4hrs. Cells were stimulated with 10ng/ml IL-3 for the indicated times. Whole cell lysates ($4x10^5$ cell equivalents) were separated be SDS PAGE on duplicate gels and immunblotted with **(C)** α- phospho-Ser^{473} or **(B)** α-phospho-Thr^{308}. Blots were stripped and reprobed with **(D)** α-PKB, **(A)** α-phospho-tyrosine and **(E)** α-myc-tag. This experiment is a repeat of that published (Craddock *et al.* 1999).

4.11 Effect of Δp85 Expression on PKB Activity

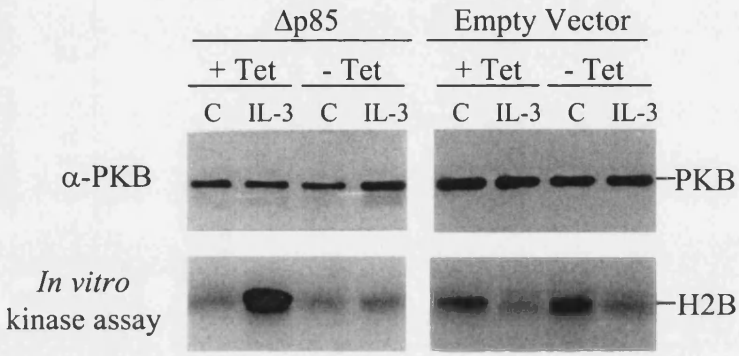
The above results (Figures 4.5 and 4.6) show that expression of the dominant negative PI3K, Δp85, inhibits the IL-3-induced phosphorylation of PKB at Ser^{473} and Thr^{308}. These two sites are phosphorylated when PKB is activated and therefore the phosphorylation of these sites is used to estimate the activity of PKB. *In vitro* kinase assays were performed to confirm that the activity of PKB was decreased in response to Δp85 expression (Figure 4.7). In the presence of tetracycline (absence of Δp85) IL-3 induced the activation of PKB but when tetracycline was removed, thus inducing the expression of Δp85, the IL-3-induced activation of PKB was inhibited. The removal of tetracycline had no effect on the activation of PKB in cells transfected with empty pUHD10-3 vector.

These data (Figures 4.5-4.7) confirm the results of the previous chapter (Figure 3.14) that demonstrate that the IL-3-induced activation of PKB in BaF/3 cells is mediated through PI3K. They identify class I_A PI3K as the isoform involved in this pathway.

4.12 Effect of Δp85 Expression on Proliferation

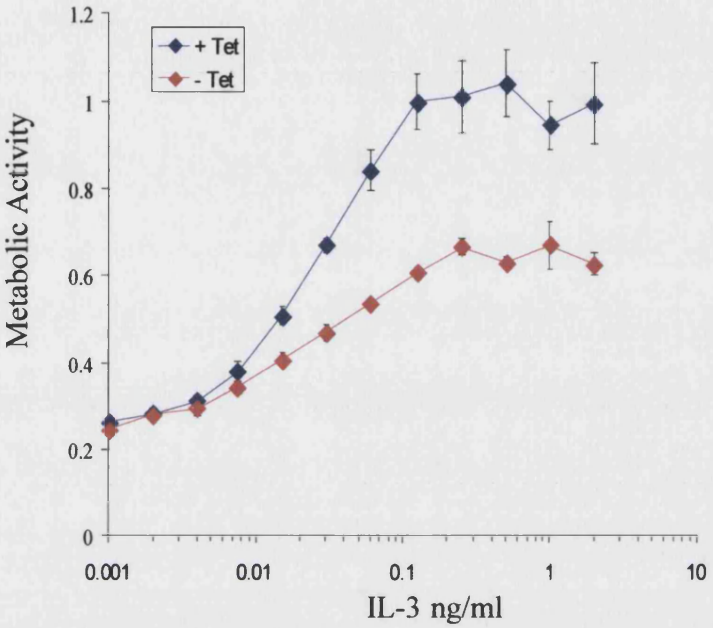
When PI3K was inhibited by LY294002 the IL-3-induced proliferation of BaF/3 cells was dramatically reduced (Figure 4.2). To investigate the effect of Δp85 expression on IL-3-induced proliferation of BaF/3 cells XTT assays were performed. Expression of Δp85 significantly reduced the proliferation in response to IL-3 (Figure 4.8A). The ED_{50} values both in the presence and absence of tetracycline remained at 0.03ng/ml indicating that only the maximum has changed and the shape of the curve remains the same. This data, together with that presented previously (Figures 4.1-4.3) illustrate the major role that PI3K plays in IL-3-driven proliferation.

Figure 4.7
Effert of Δp85 Expression on PKB Activity



Δp85 (clone 1D8) and empty vector clone were induced for 24 hours in the presence (+Tet) or absence (-Tet) of 2μg/ml tetracycline. Cells were washed free of serum and factor and starved for 1 hour prior to stimulation with 10ng/ml IL-3 for 5 min. Cells were lysed (10^7 cell equivalents) and immunoprecipitated with α-PKB. *In vitro* kinase assays were performed using histone2B (H2B) as the substrate and the products separated by SDS-PAGE and transferred to nitrocellulose. The membranes were cut in half, the upper immunoblotted with α-PKB (upper panels) and the lower subjected to autoradiography (lower panels). This experiment is representative of 3 independent experiments.

Figure 4.8
Effect of Δp85 Expression on IL-3 induced Proliferation of BaF/3 Cells



BaF/3 cells expressing Δp85 were washed free of factor and tetracycline and set up in triplicate in the presence (+) or absence (-) of tetracycline with IL-3 (at a starting concentration of 2ng/ml with serial 1:2 dilutions across a 96 well plate), or serum alone as described in materials and methods. After 72 hours the assays were developed. The mean values (n=3) with standard deviation are plotted. This experiment is representative of 3 independent experiments.

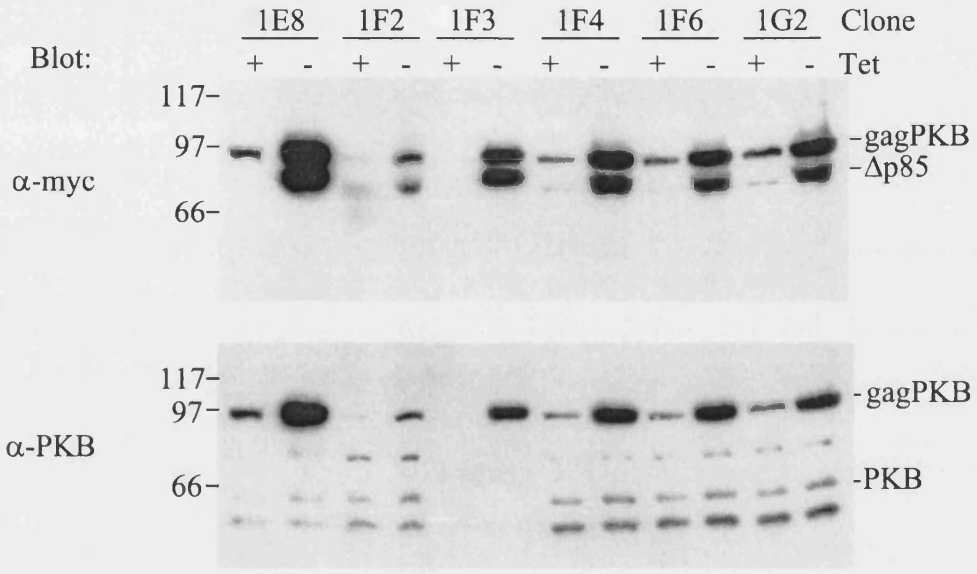
4.13 Expression of gagPKB in Δp85 expressing cells

PI3K can be thought of as a convergent point through which a number of signals from diverse origins pass on their way to a number of destinations. One such pathway travels through PKB but a number of other pathways and proteins are regulated by PI3K. To determine which effects of PI3K are mediated by PKB, a constitutively active mutant gagPKB (Burgering and Coffer, 1995) was expressed under the control of tetracycline in the Δp85 cell line. The cloning strategy employed is discussed in Section 5.3, but briefly, gagPKB was amplified by PCR from the plasmid pSG5(gagPKB) (see Table 2.3) and ligated into pBSNmyc2 to create the in-frame fusion of the myc tag epitope. The myc-tagged gagPKB cDNA was cloned into the vector pUHD10-3hygro and transfected into the Δp85 cell line. Transformed cells were selected in hygromycin and 72 resistant clones were screened for the inducible expression of gagPKB. Briefly, clones were induced over-night in the presence or absence of tetracycline, and total cell extracts immuno-blotted with anti-myc-tag and anti-PKB. An example of a screening blot is shown in Figure 4.9. Three clones, 1D12, 2C4 and 2D4, which were over-expressing gagPKB and tightly regulated by tetracycline were chosen for further analysis (Figure 4.10).

4.14 Effect of Δp85 and gagPKB expression on PKB phosphorylation

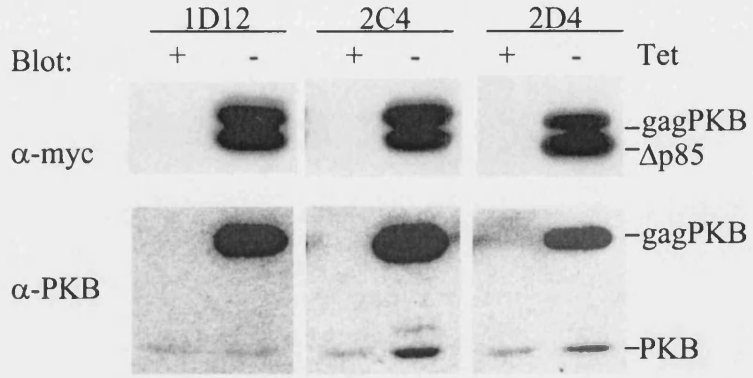
When Δp85 is expressed in BaF/3 cells the IL-3-induced proliferation and the IL-3-induced phosphorylation of endogenous PKB are dramatically decreased (Figures 4.5, 4.6 and 4.7). The main objective for creating the Δp85/gagPKB cell line was to re-introduce a signalling PKB to determine what effects of PI3K are mediated through PKB. However, before this could be examined, we first needed to ensure that gagPKB was active and to examine the effects of gagPKB expression on endogenous PKB. To do this the phosphorylation at Thr^{308} and Ser^{473} of both gagPKB and endogenous PKB were examined.

Figure 4.9
Screening blot of gagPKB in Δp85 Cells



This is an example of a screening blot. Clones were induced for 24 hours in the presence (+) or absence (-) of 2μg/ml tetracycline at a density of $1x10^5$/ml. Whole cell lysates were separated by SDS PAGE and immunoblotted with 9E10 antibody (α-myc, upper panel) which recognises the N-terminal myc tag. This gel was stripped and reprobed with α-PKB (lower panel).

Figure 4.10
Expression of gagPKB in Δp85 cells



Clones (1D12, 2C4 and 2D4) were grown for 24 hours in the presence (+) or absence (-) of 2μg/ml tetracycline at a density of $1x10^5$/ml. Whole cell lysates were separated by SDS PAGE and immunoblotted with 9E10 antibody (α-myc, upper panels) which recognises myc tag. These were stripped and reprobed with α-PKB (lower panels).

4.15 Dose-response analysis of PKB Phosphorylation

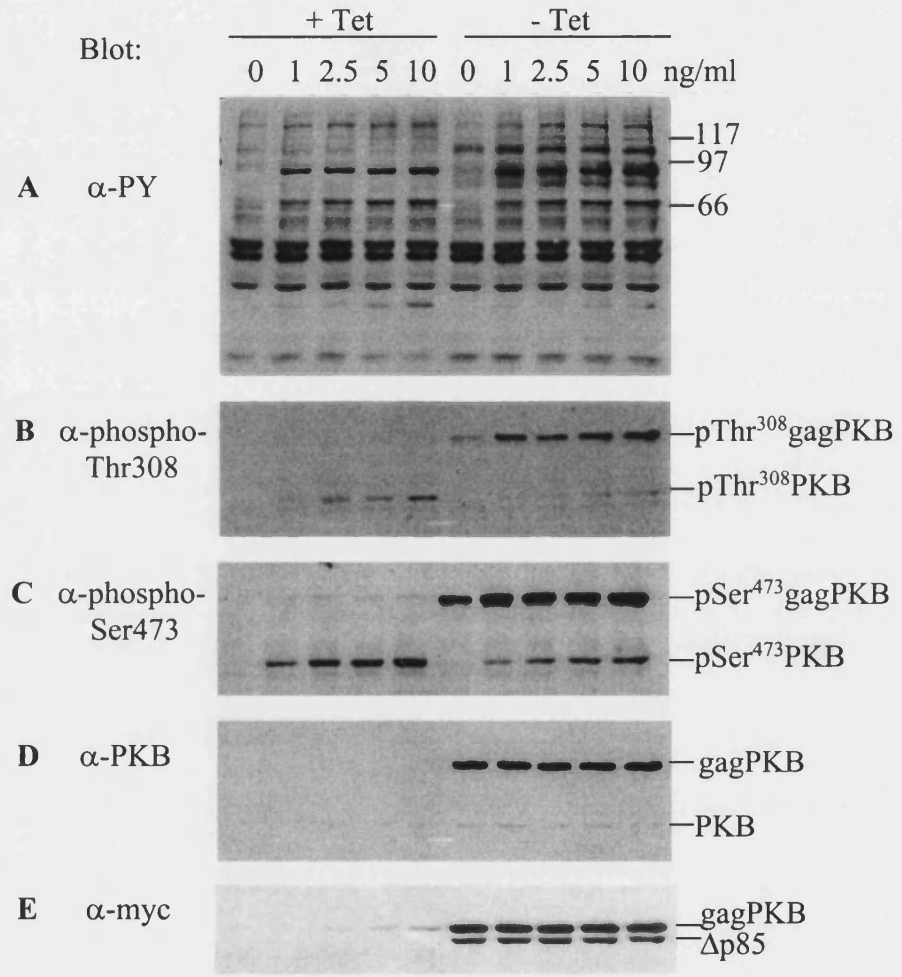
In the presence of tetracycline, IL-3 induced the phosphorylation of PKB at both Ser^{473} and Thr^{308} in a dose-responsive manner with maximal phosphorylation at 10ng/ml IL-3. This correlates with the maximal phosphorylation observed in untransfected BaF/3 cells (Figure 3.10). The removal of tetracycline and subsequent expression of Δp85 and gagPKB led to a decrease in the phosphorylation of endogenous PKB at both Thr^{308} and Ser^{473} (Figure 4.11). However, when the level of phosphorylation in the presence of both gagPKB and Δp85 are compared to the level seen when Δp85 only is expressed, there is a significant increase in phosphorylation of endogenous PKB (Figure 4.5 and 4.10). A high level of basal gagPKB phosphorylation at Ser^{473} was observed and this phosphorylation was increased by stimulation with IL-3.

4.16 Kinetic analysis of PKB Phosphorylation

When the expression of Δp85 and gagPKB was repressed by the presence of tetracycline IL-3 stimulated the phosphorylation of Ser^{473} and Thr^{308}, with maximal phosphorylation observed at 5-10 minutes (Figure 4.12). In the absence of tetracycline when Δp85 and gagPKB were expressed, the IL-3-induced phosphorylation of endogenous PKB was reduced. However, as seen in the dose-response analysis (Figure 4.8), levels of PKB phosphorylation are increased when compared to those seen when Δp85 only is expressed (Figures 4.6 and 4.11). The level of basal gagPKB Thr^{308} phosphorylation is lower than that seen in the dose response analysis, the reason for this is unclear. However, gagPKB phosphorylation at both Ser^{473} and Thr^{308} is increased by treatment with IL-3.

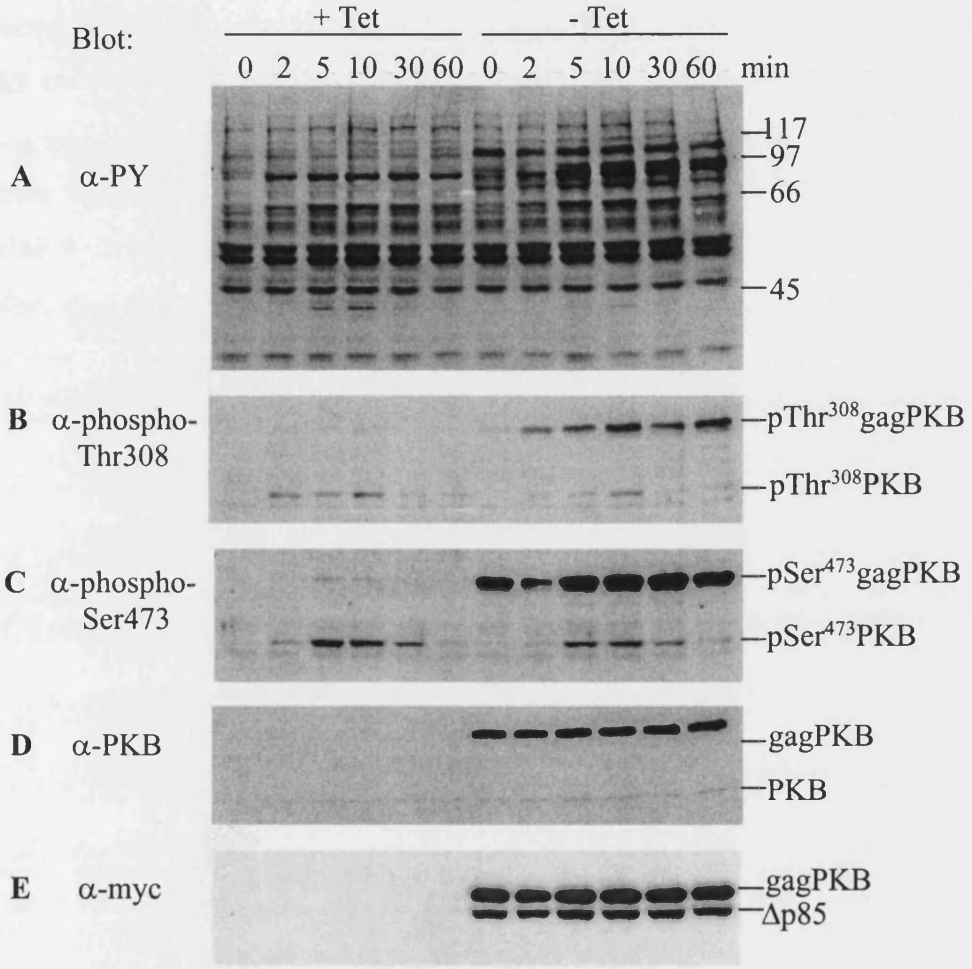
Phosphorylation at Thr^{308} and Ser^{473} are required for maximum activation of PKB. In addition to this, results in the previous chapter found a strong correlation between phosphorylation at Ser^{473} and PKB activity. This evidence provides support to the theory that gagPKB is constitutively active and acts in a dominant positive manner. Additionally, *in vitro* kinase assays indicate that gagPKB that is expressed alone in BaF/3 cells has a low basal level of activity (Figure 5.3). The expression of gagPKB also restores the phosphorylation, and presumably, the activity of endogenous PKB. Therefore, the Δp85/gagPKB cell line provides us with a system whereby PI3K pathways have been inhibited but PKB mediated pathways have been reinstated. This provides a useful cell model in which to investigate the role of PKB in PI3K mediated events.

Figure 4.11
Effect of Δp85 and gagPKB expression on PKB phosphorylation: Dose-response analysis



Cells were set up at 10^5/ml in the absence (- Tet) or presence (+ Tet) of tetracycline for 24hr prior to starving of IL-3 and serum for 4hrs. Cells were stimulated for 5 minutes with the indicated concentration of IL-3. Whole cell lysates ($4x10^5$ cell equivalents) were separated by SDS PAGE on duplicate gels and immunblotted with α-phospho-Ser^{473} **(C)** or α-phospho-Thr^{308} **(B)**. Blots were stripped and reprobed with α-PKB **(C)**, α-phospho-tyrosine **(A)** and α-myc-tag **(E)**. This experiment is representative of 3 independent experiments.

Figure 4.12
Effect of Δp85 and gagPKB expression on PKB phosphorylation: Kinetic analysis



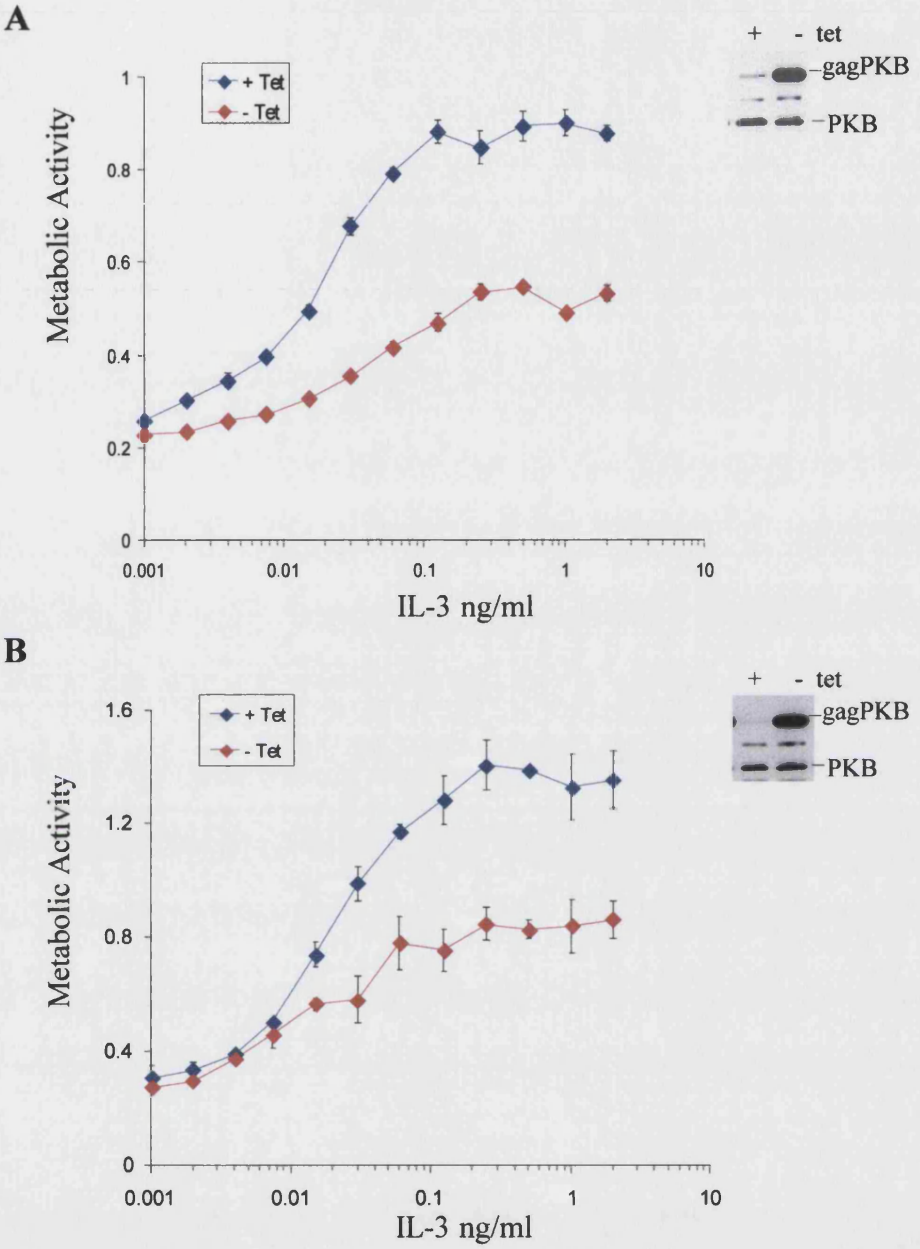
Cells were set up at 10^5/ml in the absence (- Tet) or presence (+ Tet) of tetracycline for 24hr prior to starving of IL-3 and serum for 4hrs. Cells were stimulated with 10ng/ml IL-3 for the indicated times. Whole cell lysates ($4x10^5$ cell equivalents) were separated be SDS PAGE on duplicate gels and immunblotted with α- phospho-Ser^{473} **(C)** or α-phospho-Thr^{308} **(B)**. Blots were stripped and reprobed with α-PKB **(D)**, α-phospho-tyrosine **(A)** and α-myc-tag **(E)**. This experiment is a representative of 3 independent experiments.

4.17 Effect of Δp85 and gagPKB expression on proliferation

The major effect of PI3K inhibition in BaF/3 cells was a reduction in IL-3-induced proliferation (Figures 4.2 and 4.7). Given that PKB is activated through PI3K and that PKB is reported to play a role in proliferation in IL-2 signalling (Brennan *et al.*, 1997; Ahmed *et al.*, 1997) it was interesting to see if expression of gagPKB was sufficient to overcome the inhibitory effect of Δp85 expression on proliferation (Figure 4.8). XTT dye-reduction assays showed that gagPKB expression was unable to restore the IL-3-induced proliferation of BaF/3 Δp85 (1D8) cells to the levels observed in the absence of Δp85 (Figure 4.13). ED_{50} values in the presence and absence of tetracycline were 0.2ng/ml and 0.4ng/ml IL-3 respectively.

These data demonstrate that PKB is not required for the PI3K-mediated proliferation of BaF/3 cells, indicating that either another pathway downstream of PI3K is involved or that the gagPKB construct used is unable to reconstitute the PKB signal which is inhibited in Δp85 cells.

Figure 4.13
Effect of Δp85 and gagPKB Expression on IL-3-Induced Proliferation of BaF/3 Cells



BaF/3/Δp85 cells expressing gagPKB, **(A)** clone 1D12 and **(B)** clone 2C4, were washed free of factor and tetracycline and set up in triplicate in the presence (+) or absence (-) of tetracycline with IL-3 (at a starting concentration of 2ng/ml with serial 1:2 dilutions across a 96 well plate), or serum alone as described in materials and methods. After 72 hours the assays were developed. The mean values (n=3) with standard deviation are plotted. **Insert**: At the same time that the XTT assays were set up, cells were grown +/- tetracycline for 72 hours. Cells extracts were separated by SDS-PAGE and immunoblotted with α-PKB to see protein expression. This experiment is representative of 3 independent experiments.

4.18 Discussion

The experiments presented in this chapter used the tetracycline-regulated inducible gene expression system to express a dominant negative PI3K and a constitutively active PKB mutant in BaF/3 cells. The major effect of PI3K inhibition observed in these cells was an inhibition of the IL-3-mediated proliferation (Figure 4.1, 4.2 and 4.7). Attempts to reverse the negative effect of PI3K inhibition on proliferation by reconstituting the PKB signal in these cells proved unsuccessful (Figure 4.13). This suggests that either PKB is not involved in the PI3K mediated proliferative pathway or the PKB mutant used is unable to successfully reconstitute the PKB signalling pathway.

The PKB mutant used in this study is based on the oncogenic *v-Akt*, which is made up of a gag fusion (consisting of p12, p15 and a truncated p30), a 21 amino acid region of cellular PKB untranslated sequence and cellular PKB (Bellacosa *et al.*, 1991). v-Akt is myristylated at its N-terminal glycine and this is thought to target it to the cellular membrane, where presumably it is constitutively phosphorylated and active (Ahmed *et al.*, 1993). Indeed, *v-Akt* has a cellular distribution of 40% plasma membranes 30% nuclear and 30% cytosolic, while PKB is 90% cytosolic (Ahmed *et al.*, 1993). The gagPKB used in this and other studies (Burgering and Coffer, 1995) was constructed by fusing a MoMuLV cDNA fragment encoding the gag proteins to the N-terminus of PKB. It is presumed that this fusion protein is processed to give a myristylated N-terminal glycine residue that targets it to the plasma membrane. However, the gagPKB construct used in this study has a myc tag attached to its N-terminus to aid in the detection of the mutated protein. Therefore, the myc-gagPKB does not have an N-terminal glycine residue, which is required for myristolation. Cellular distribution studies of the myc-tagged gagPKB show that only 5% is located at the membrane in unstimulated cells (C. Beck and M.J. Welham, unpublished data). However, in unstimulated BaF/3 cells when co-expressed with Δp85, gagPKB is phosphorylated on both Ser^{473} and Thr^{308} (Figures 4.11 and 4.12). Phosphorylation at both of these sites is required for maximal activation of PKB and work in the previous chapter (Figures 3.1-3.15) showed that the phosphorylation of Ser^{473} correlates with the activity of PKB. This suggests that despite not being targeted to the membrane at high levels, gagPKB is active in unstimulated Δp85 expressing cells. These data suggest that either membrane association is not important for the phosphorylation of gagPKB in the absence of PI3K activity or gagPKB associates transiently with the membrane resulting in its phosphorylation.

In the viral life cycle gag proteins are involved in directing the budding process as well as being structural components of the virion. Gag mutants that are not myristylated are defective at both processes and this is thought to be due to decreased membrane association (Shultze and Rein, 1989). However, some gag proteins are not myristylated (ERDIE, Wills, 1990) and myristic acid on its own does not provide enough binding energy to anchor proteins to the lipid bilayer (Mclaughlin and Aderem, 1995). While the membrane association of gag mutants that are defective at mysitolation is decreased it is not completely abolished (Huang and Jolicoeur, 1994). It is thought that amino acid residues close to N-terminus also play a role in anchoring gag proteins to membrane (Verderame *et al.*, 1996; Zhou *et al.*, 1994). Therefore, it is possible that the gag fusion enables gagPKB to associate with membranes in a transient manner resulting in its phosphorylation at Ser^{473} and Thr^{308} even in the absence of myristolation.

Alternatively, membrane association may be less important for the phosphorylation of PKB in the absence of PI3K activity. PDK-1 is constitutively active and exists in the cytosol in unstimulated cells (Alessi *et al.*, 1997; Pullen *et al.*, 1998; Anderson *et al.*, 1998; Casamayor *et al.*, 1999). Increased levels of PIP_3 are believed to target PDK-1 to the membrane where it interacts and phosphorylates PKB on Thr^{308}. In the absence of PIP_3, and when gagPKB is over-expressed, membrane targeting may not be required for the interaction of PKB and PDK-1 and the subsequent phosphorylation.

The activity of the gagPKB mutant could be confirmed by a number of methods. Firstly, the effect of its expression on the activity/phosphorylation of downstream targets of PKB could be examined. For instance, the activation of GSK3α and β, or the phosphorylation of Bad on Ser^{136} could be examined. Another method would be to look at the ability of the gagPKB construct to transform cell lines. *v-Akt* was identified as an oncogene, and therefore one would expect that an active gagPKB mutant should also act as an oncogene. This could be tested by transfecting 3T3 fibroblast cells with the gagPKB construct to see how many cells are transformed, as judged by morphological changes. Alternatively, retroviral transformation could be used in which case we would expect a high proportion of cells to be transformed.

Expression of Δp85 in BaF/3 cells resulted in a marked reduction in the IL-3-induced phosphorylation and activation of PKB (Figures 4.5-4.7). When gagPKB is also expressed in these cells the Δp85-induced inhibition of endogenous PKB phosphorylation is partially reversed, particularly in respect to Ser^{473} (Figures 4.10 and 4.11). Datta *et al.* (1995) and Franke *et al.* (1997) reported that PKB can form

oligomers through the interaction of their PH domains. This could explain the increased phosphorylation of endogenous PKB that we observed when gagPKB was expressed in BaF/3/Δp85 cells. The formation of a gagPKB-endogenous PKB oligomer in these cells would allow the endogenous PKB to associate with the membrane in the absence of PIP_3. This would allow the phosphorylation and activation of endogenous PKB by the same mechanism that activates membrane targeted PKB mutants.

A major effect of PI3K inhibition observed in BaF/3 cells was the inhibition of IL-3-induced proliferation (Figures 4.2 and 4.5). To examine the role of PKB in this PI3K/proliferative pathway a constitutively active PKB mutant was transfected into BaF/3/Δp85 cells (Figures 4.6 and 4.7). This gagPKB mutant was shown to be phsophorylated on Ser^{473} and Thr^{308} even in the absence of IL-3 (Figures 4.11 and 4.12). Phosphorylation at both these sites is required for the maximal activtation of PKB and therefore can be used as an indication of PKB activity. In addition, gagPKB was shown to have a low basal level of activity when transfected alone into BaF/3 cells (Figure 5.3). These data suggest that gagPKB is constitutively active in this BaF/3/Δp85 cell line. However, the re-introduction of an active PKB (gagPKB) into this PI3K compromised cell line did not reverse the proliferative defect (Figure 4.13). This suggests that either PKB is not involved in the IL-3-induced proliferation of BaF/3 cells, or that gagPKB is unable to functionally couple to the downstream effectors of this pathway.

A number of PKB substrates exist primarily in the cytosol or nucleus and this raises the concern that membrane bound PKB, while being constitutively active, may be unable to couple to physiologically relevant substrates at other locations. The gagPKB mutant used in this work is not always at membrane. Therefore, it should be free to interact with substrates at locations other than the membrane. However, the possibility that the myc-gag fusion causes conformational changes that prevent the association of this mutant with its substrates cannot be ruled out.

If we accept that the gagPKB mutant is able to restore signalling from PKB, then the results in Figure 4.13 indicate that PKB is not involved in the IL-3-induced, PI3K mediated proliferation of BaF/3 cells. Therefore, other PI3K mediated pathways must be involved. Two additional pathways that have been implicated in proliferation and are regulated by PI3K are the MAPK pathway (Cross *et al.*, 1994; Ferby *et al.*, 1994) and the p70S6 kinase pathway (Weng *et al.*, 1995). Recent work in this laboratory has found that the activation of the MAP kinases, Erk1 and Erk2, is inhibited by the

expression of Δp85 and that the Mek inhibitor PD98059 reduced the IL-3-induced proliferation of BaF/3 cells (Craddock *et al.*, in press). This suggests that the PI3K and Mek mediated activation of Erk1 and Erk2 may be involved in the IL-3-driven proliferation of BaF/3 cells. However, the combination of PD98059 and Δp85 expression further reduced the IL-3-driven proliferation, which suggests that other PI3K mediated pathways are also involved. There is some evidence to suggest that PI3K may be modulating the levels and function of cell cycle regulators. In BaF/3 cells, treatment with LY294002 resulted in a decrease in phosphorylation of the pocket proteins p107, p130 and pRB, in addition to a decrease in overall cyclinD3 levels (B. Fox and M.J. Welham, unpublished data).

The PKB/PI3K pathway has been widely implicated in the control of apoptosis (reviewed in Coffer and Woodgett, 1998; Datta *et al.*, 1999, Vanhaesebroeck and Alessi, 2000). However, the inhibition of PI3K by LY294002 and the subsequent inhibition of PKB did not significantly affect the number of BaF/3 cells undergoing apoptosis (Figure 4.3). This result correlates with previous work where Δp85 expression in BaF/3 cells resulted in only a 3% increase in apoptosis (Craddock *et al.*, 1999). One downstream effector of PKB in the apoptotic pathway is the Bcl-2 family member Bad. PKB is reported to phosphorylate Bad on Ser^{136}, which decreases its interaction with Bcl-2, with the net effect of decreasing apoptosis (Datta *et al.*, 1997). Bad is also serine phosphorylated at other sites, and its hyper-phosphorylation results in a shift in its migration on SDS PAGE. The results presented in Figure 3.15C showed that pre-treatment of BaF/3 cells with LY294002 completely inhibited the shift in Bad migration indicating that it is not hyper-phosphorylated, nor is it phosphorylated at Ser^{112}. Migration shifts only give an indication of the overall level of phosphorylation as both phosphorylated and unphosphorylated Bad migrate in the lower band (Zha *et al.*, 1996). However, in the presence of LY294002, PKB activation (Figure 3.14) and Bad hyper-phosphorylation (Figure 3.15C) were inhibited, yet apoptosis was not increased (Figure 4.3). This suggests that in IL-3-dependent BaF/3 cells the PI3K/PKB/Bad pathway is not necessary to provide a survival signal.

The effect of gagPKB expression on Bad phosphorylation in the BaF/3Δp85 cells was not examined. Scheid *et al.* (1999) have reported that the PKB phosphorylation site on Bad, Ser^{136}, was not phosphorylated in response to GM-CSF in MC9 cells. This suggests that this site is not physiologically relevant to GM-CSF signalling. As IL-3 and GM-CSF share the β_c receptor subunit and many signalling pathways, it would be

interesting to investigate the phosphorylation status of Bad in the Δp85/gagPKB cells. As LY294002 treatment or Δp85 expression was sufficient to inhibit the hyper-phosphorylation of Bad, it would be interesting to investigate the effects of gagPKB expression, when PI3K is inhibited, on the phosphorylation of Bad.

The Δp85/gagPKB cell line generated in this work provides an ideal cell model, in which to specifically investigate the PI3K dependent pathways that are mediated through PKB. Many studies in the past have looked at the correlation between PKB inhibition by PI3K inhibitors or dominant negative PI3K variants, and downstream effects. However, this approach does not distinguish between PKB mediated events and those that are mediated by other targets of PI3K. Therefore it would be interesting to look at the effects of PI3K inhibition coupled with a constitutively active PKB variant on PKB substrates, for example GSK3 and the forkhead proteins.

The results presented in this chapter used both pharmacological inhibitors and a dominant negative PI3K to demonstrate that PI3K plays an important role in the IL-3 mediated proliferation of BaF/3 cells. The role of the downstream effector of PI3K, PKB in IL-3-driven proliferation was also examined. By expressing a constitutively active PKB variant, which is not dependent on PI3K for its activity, in Δp85 cells, we were able to reconstitute PKB signalling pathways and demonstrate that PKB is not sufficient to overcome the loss of PI3K and restore normal proliferation.

Chapter 5

The Effects of PKB Variants Expression on IL-3 Signalling

5.1 Introduction

The overall aim of this project was to investigate the role of PKB in cytokine signalling in haemopoietic cells. The early stages of this work (see chapter 3) found that all the cytokines tested were able to induce the activation of PKB in the cytokine dependent cell lines examined (BaF/3, FD-6 and MC9). The activation of PKB was found to be dependent on the integrity of PI3K signalling pathways as the inhibition of PI3K significantly reduced the cytokine-induced activation of PKB. At the time of this work PKB was widely reported to be involved in the control of apoptosis (see Coffer and Woodgett, 1998; Datta *et al.*, 1999; Vanhaesebroeck and Alessi, 2000 for a review). However, when the activation of PKB was compared with the phosphorylation of Bad and the ability of a particular cytokine to maintain the viability of these cell lines a definite correlation was not found (Chapter 3). These data raised questions about the role that PKB plays in the prevention of apoptosis in these cell lines.

The experiments performed in Chapter 3 used the PI3K inhibitor LY294002 to inhibit PKB activity and did not directly examine the effects of PKB inhibition. PI3K regulates many pathways and it is possible that the inhibition of these other pathways masked the effects of PKB inhibition. As there are no specific pharmacological inhibitors of PKB available, PKB mutants were used to specifically investigate the role of PKB in cytokine signalling.

The tetracycline-regulated gene expression system (section 4.7) was used to express PKB variants in BaF/3 cells. This system was chosen because it allows the expression of mutant proteins to be tightly regulated. This was deemed important as PKB is reputed to be involved in apoptosis and proliferation, therefore interruption of this pathway could be lethal to the cells resulting in the inability to derive transfectants. A number of PKB mutants were chosen for this study. They were:

- gagPKB – reputed to be a dominant positive PKB mutant, which was modelled on the constitutively active *akt* oncogene product (Burgering and Coffer, 1995).
- cxPKB – reputed to be a dominant negative PKB mutant that is membrane bound by a C-terminal *Ki ras* caax sequence (van Weeren *et al.*, 1998).
- kdPKB – reputed to be a dominant negative PKB mutant that is rendered kinase inactive by the point mutation K-A^{179} in the active site (Burgering and Coffer, 1995).
- wtPKB - wild-type PKB (Burgering and Coffer, 1995).

5.2 The Effects of PKB Variants on IL-3 Signalling

By expressing these PKB variants in BaF/3 cells we hoped to create model cell systems in which PKB activity and signalling was either specifically inhibited or independent of IL-3 stimulation. The gagPKB mutant, which is modelled on *v-Akt*, was chosen for its dominant positive effects. This oncogenic form of PKB is rendered constitutively active by the translocation of a viral gag protein to its N-terminus. This targets the *v-Akt* to the cell membrane where it is maintained in a constitutively phosphorylated and active state. The gagPKB mutant used in these studies is homologous to *v-Akt* and has been shown to act in a dominant positive manner when transiently transfected in Rat1 cells (Burgering and Coffer, 1995).

To inhibit PKB mediated pathways two putative dominant negative variants were used. The first was a kdPKB that is rendered catalytically inactive by the point mutation of Lys^{179} to Ala in the active pocket of the kinase domain and was reported to have a dominant negative effect on endogenous PKB in a number of cell systems (Datta *et al.*, 1997; Kulik and Weber, 1998; Takata *et al.*, 1999). However, in other systems it does not act as a dominant negative (Burgering and Coffer, 1995). Therefore, the effectiveness of kdPKB at inhibiting endogenous PKB activation appears to be dependent on the system used. Because of the apparent variability in the effects of kdPKB between systems a second putative dominant negative mutant (cxPKB) was also expressed in BaF/3 cells. This mutant was originally constructed to be a dominant positive PKB but when it was expressed it was found to act in a dominant negative manner. cxPKB is targeted to the cell membrane via a C-terminal caax sequence (KMSKDGKKKKKKSKTKCVIM) from Ki-*ras* (van Weeren *et al.*, 1998). The -aax motif (see underlined, a = aliphatic, x = any amino acid) is removed to give a C-terminal cysteine residue. This residue undergoes methylesterification, which helps to target it to the membrane. However, this processing alone is not sufficient for membrane association and the poly-basic charge of the 6 consecutive lysine residues is also required for efficient membrane association (Hancock *et al.*, 1990). It was thought that by targeting cxPKB to the membrane this would render it constitutively phosphorylated and therefore activated. However, this was not the case and cxPKB was found to act in a dominant negative manner (van Weeren *et al.*, 1998). The reason for this is not clear, but it may be that both the N- and C- termini of cxPKB can interact with the membrane. This may cause a conformational change that could prevent cxPKB's activation by phosphorylation, interrupt the active site or prevent substrate access.

The final PKB variant used in these studies was the wild-type PKB. This mutant was identical to cellular PKB with the exception of an N-terminal myc tag and being under the control of tetracycline. wtPKB was included primarily as a control, and while it was expected that over-expression of this protein would have effects on PKB signalling, it would always be dependent on IL-3 for activity.

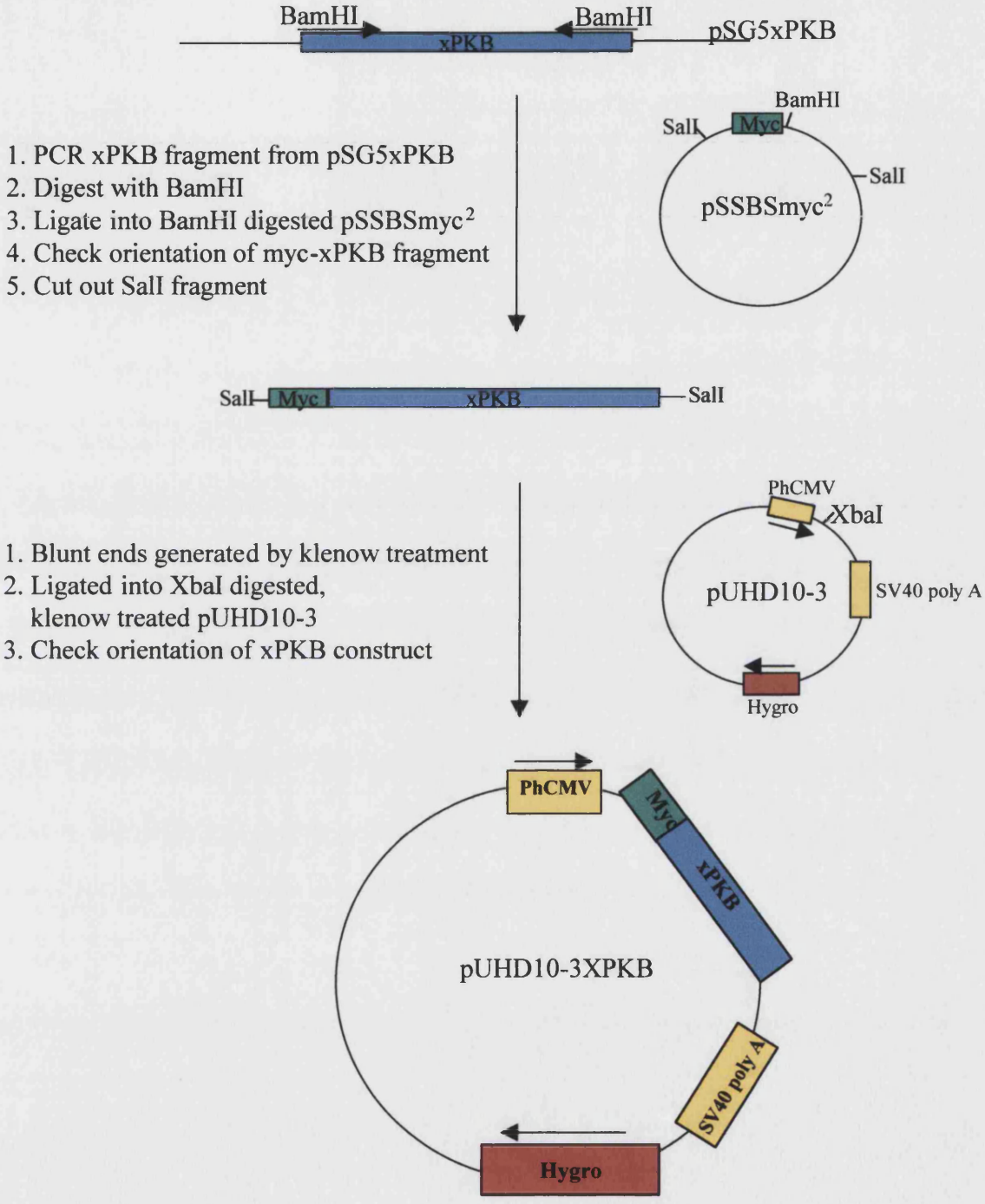
5.3 Construction of pUHD10-3hygro Expression Vectors.

The plasmids expressing the PKB variants that were used for the construction of pUHD10-3hygro vectors were a kind gift from Boudewijn M. Th. Burgering (Laboratory for Physiological Chemistry, Utrecht University, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands) and are listed in Table 2.3. A two-step strategy was used for the construction of the expression vectors and is outlined in Figure 5.1. The gene of interest was amplified from the original pSG5 plasmid by PCR. The primers (which are listed in Table 2.2) contained BamHI restriction sites to create a restriction site at each end of the PCR fragment. The PCR fragment containing the PKB mutant was restriction digested with BamHI and ligated into the vector pSSBS.myc2. This generated an in-frame fusion of two tandem copies of the 10 amino acid myc epitope tag to the N-terminus of the PKB mutants. Myc tag epitopes are recognised by the 9E10 antibody. Colonies were screened for vectors containing the PKB inserts in the correct orientation. myc-PKB fragments were digested with SalI, blunt-ended by klenow treatment and ligated into XbaI digested and klenow treated pUHD10-3hygro.

5.4 Expression of PKB Mutants in BaF/3 Cells.

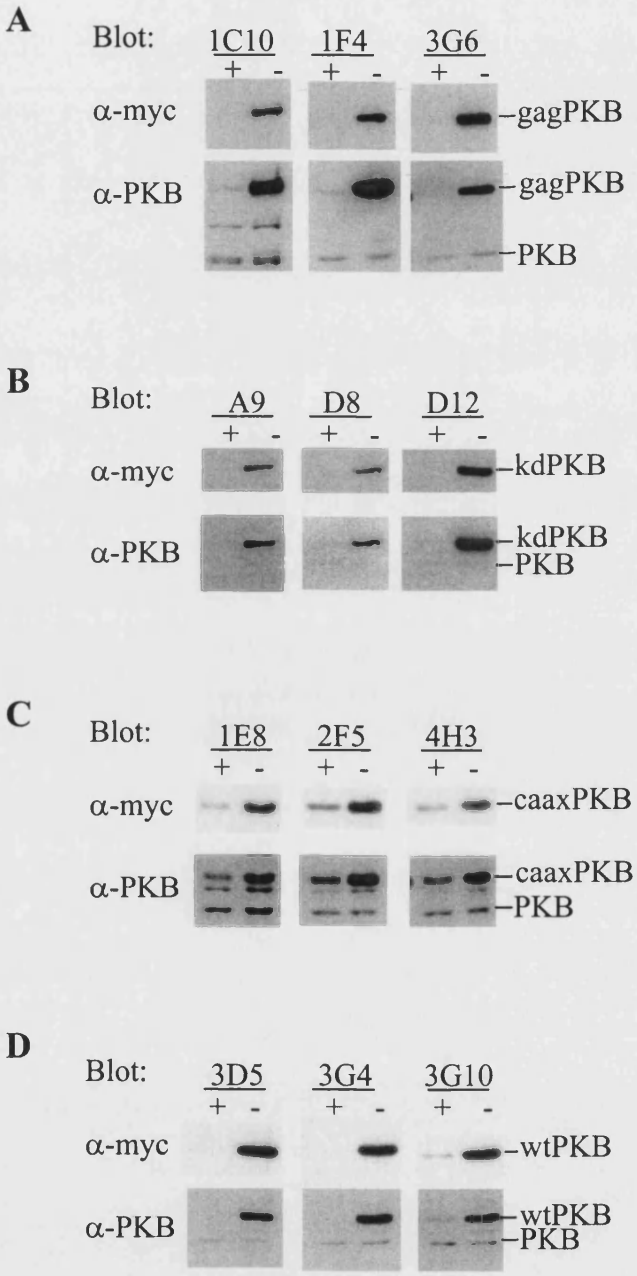
Each of the PKB mutants (gagPKB, cxPKB, kdPKB and wtPKB) were transfected into BaF/3 cells that had been stably transfected with the regulator plasmid pUHD15-1 (Mui *et al.*, 1995). Transformed cells were selected in hygromycin and at least 72 resistant clones for each PKB mutant were screened in the presence and absence of tetracycline for exogenous PKB expression. Three clones for each mutation, which over-expressed the exogenous protein and had low levels of expression in the presence of tetracycline, were chosen for further analysis. These clones are shown in Figure 5.2.

Figure 5.1
Cloning of PKB Variants into pUHD10-3



The cloning steps involved in the construction of the pUHD10-3 expression vectors are outlined above. The same strategy was taken with each PKB variant and xPKB refers to the coding sequence of all the PKB variants.

Figure 5.2
Expression of PKB Variants in BaF/3 Cells

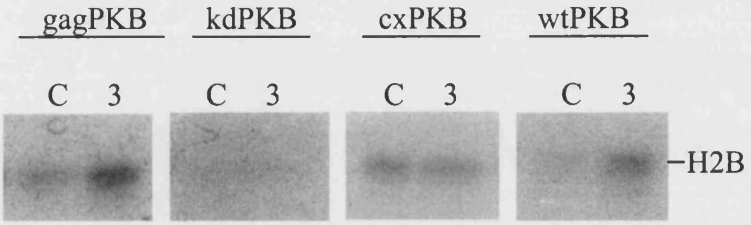


(A) gagPKB clones, **(B)** kdPKB clones, **(C)** cxPKB clones and **(D)** wtPKB clones were grown for 24 hours in the presence (+) or absence (-) of 2μg/ml tetracycline at a density of approximately $1x10^5$/ml. Whole cell lysates were separated by SDS PAGE and immunoblotted with 9E10 antibody (α-myc, upper panel) which recognises myc tag. These were stripped and reprobed with α-PKB (lower panels).

5.5 Activity of PKB Mutants

To examine the activity of each PKB mutant, clones were either stimulated with IL-3 or left untreated. Exogenous PKB was immunoprecipitated with 9E10 antibody that recognises the N-terminal myc tag of each mutant and their ability to catalyse the phosphorylation of histone2B was examined by *in vitro* kinase assays (Figure 5.3). kdPKB was inactive both before and after stimulation with IL-3 indicating that, as expected, it is kinase inactive. The activity of both wtPKB and gagPKB was dramatically increased by treatment with IL-3. However, when the level of basal activity of the wtPKB and gagPKB are compared, the level of gagPKB activation is significantly higher, reflecting a high level of constitutive kinase activity that is increased upon IL-3 stimulation. The activation of cxPKB was constitutively high and independent of IL-3 stimulation, which was surprising given that van Weeren *et al.* (1998) found that the activity of cxPKB was comparable to that of unstimulated wtPKB in both unstimulated and insulin stimulated A14 cells. cxPKB is reputed to work in a dominant negative manner but the mechanism is not understood. One theory is that the caax domain anchors cxPKB to the membrane by its C-terminus while the PH domain targets the N-terminal domain to the membrane. This is thought to cause a conformational change in the enzyme thereby preventing its kinase activity. Before immunoprecipitation, cells are lysed interrupting the cell membrane structure and proteins are released from the membrane. It is feasible that this process allows cxPKB to regain its kinase activity, as it is no longer tethered to the membrane at both the C- and N-termini. This could explain the constitutive activation of cxPKB seen in *in vitro* kinase assays (Figure 5.3).

Figure 5.3
Activity of PKB Variants in BaF/3 Cells



The expression of PKB variants was induced for 24 hours by the removal of tetracycline. Cells were starved of serum and factor for 1 hour before stimulation for 10 minutes with 10ng/ml IL-3 (3) or left unstimulated (C). PKB variants were immunoprecipitated with 9E10 and *in vitro* kinase assays performed using histone2B (H2B) as the substrate. These were separated by SDS PAGE, transferred to nitrocellulose and subjected to autoradiography. This experiment is representative of 3 independent experiments.

5.6 The Effects of PKB Variants on PKB Phosphorylation

Having examined the activity of each of the PKB variants, it was then important to investigate the effects their expression was having on endogenous PKB activity. To do this the phosphorylation of endogenous PKB at Ser^{473} and Thr^{308} was examined, as results from *in vitro* kinase assays would be masked by the activity of the exogenous protein. Phosphorylation at both of these sites is required for the maximal activation of PKB (Alessi *et al.*, 1996) and previous work found that the phosphorylation of Ser^{473} closely mimicked the activity of PKB (Figures 3.1-3.5). Initially, the activation of endogenous PKB was examined in one clone for each PKB variant.

The gagPKB construct used in these experiments was modelled on the oncogenic *v-Akt*. It had been reported to be constitutively active and by analogy to *v-Akt*, it was thought that this was due to its constitutive phosphorylation. The effects of gagPKB expression on endogenous PKB were largely unknown, but it was not expected that it would have a marked effect on endogenous PKB activity. However, the expression of gagPKB in BaF/3/Δp85 cells was able to partially reverse the inhibitory effect of Δp85 expression on the phosphorylation of endogenous PKB (compare Figures 4.5 and 4.6 with Figures 4.11 and 4.12). This was a surprising result and suggests that in addition to being constitutively active, gagPKB may be acting as a dominant positive by increasing the activity of endogenous PKB.

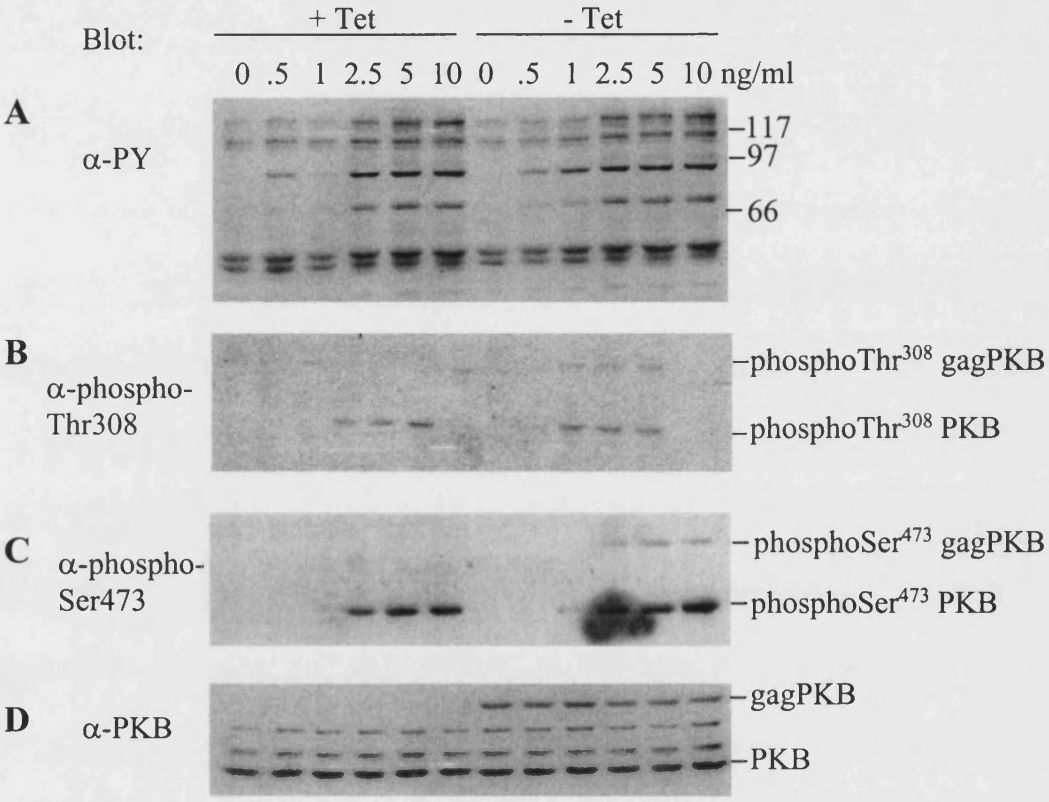
To investigate the effects of gagPKB expression alone on PKB activation in BaF/3 cells, the phosphorylation of both Ser^{473} and Thr^{308} were examined in clone 1C10 (Figure 5.4). In the presence of tetracycline, when the expression of gagPKB is repressed, IL-3 induced the phosphorylation of endogenous PKB on both Ser^{473} and Thr^{308} in a dose responsive manner. However, the expression of gagPKB alone does not increase the phosphorylation of endogenous PKB as was observed when gagPKB was expressed in BaF/3/Δp85 cells (Figure 4.10).

The phosphorylation of gagPKB on Ser^{473} and Thr^{308} is also induced by IL-3 stimulation. However, no basal phosphorylation of gagPKB is detected. Again this is in contrast to results seen when both Δp85 and gagPKB are expressed (Figure 4.10) and to the results of gagPKB *in vitro* kinase assays (Figure 5.3), which showed a constitutive level of activation. The reasons for this are not clear but it should be noted that the level of exogenous gagPKB expression in this experiment is not as high as that seen in Figure 4.10. Alessi and colleagues recently reported that basal Ser^{473} phosphorylation is markedly increased in PDK-1 -/- ES cells (Williams *et al.*, 2000).

Thus, inhibition of the PI3K/PDK-1 pathway may act to increase Ser^{473} phosphorylation.

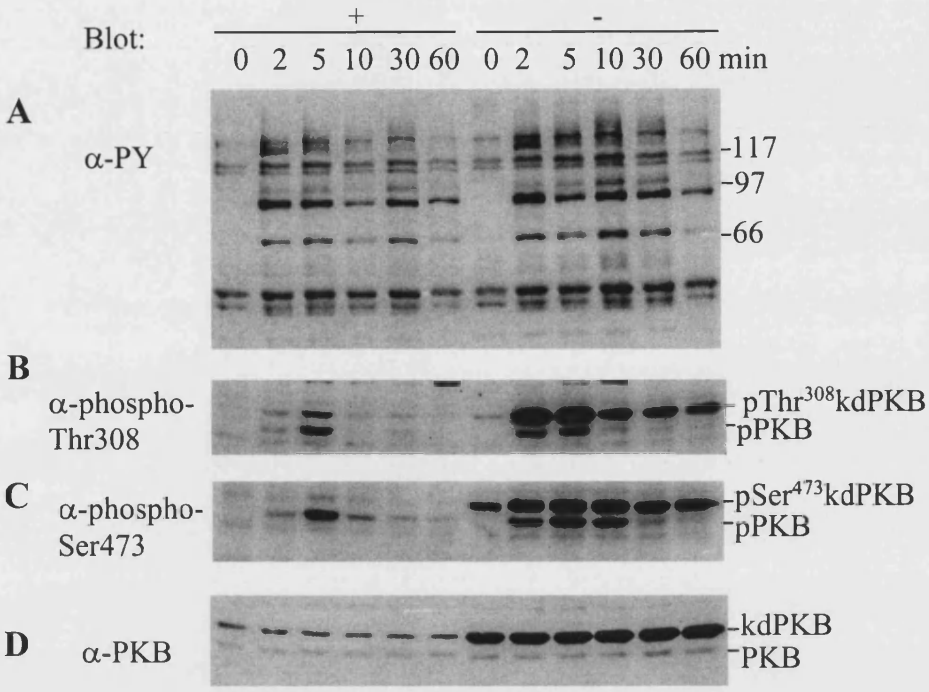
Both kdPKB and cxPKB have been reported to have a dominant negative effect on PKB. Therefore, we would expect that expression of these mutants would inhibit the phosphorylation of both Ser^{473} and Thr^{308} on endogenous PKB. Figure 5.5 shows the effect of kdPKB expression on the phosphorylation of PKB in clone D12. In the absence of tetracycline when kdPKB is expressed the phosphorylation of both Ser^{473} and Thr^{308} is increased compared to in the presence of tetracycline. When these blots were stripped and reprobed with pan-PKB it is apparent that there is marginally more protein loaded in the - tetracycline lanes, however, this could not account for the magnitude of increase in phosphorylation observed. It is also interesting to note the high level of kdPKB phosphorylation at Ser^{473} and Thr^{308} in particular in the unstimulated control lanes, although as kdPKB is catalytically inactive (Figure 5.3) this has no impact on its activity. This is a surprising result, as in other systems kdPKB does not affect the activity of endogenous PKB. kdPKB is thought to act in a dominant negative fashion by competing with endogenous PKB to prevent activation of downstream targets. Therefore, the effects of kdPKB expression on PKB substrates must also be investigated.

Figure 5.4
Effect of gagPKB expression on PKB phosphorylation
Dose Response analysis



gagPKB cells were set up at 10^5/ml in the absence (- Tet) or presence (+ Tet) of tetracycline for 24hr prior to starving of IL-3 and serum for 4hrs. Cells were stimulated for 5 minutes with the indicated concentration of IL-3. Whole cell lysates ($4x10^5$ cell equivalents) were separated be SDS PAGE on duplicate gels and immunblotted with α- phospho-Ser^{473} (C) or α-phospho-Thr^{308} (B). Blots were stripped and reprobed with α-PKB (D) and α-phospho-tyrosine (A). This experiment is a representative of 2 independent experiments.

Figure 5.5
Effect of kdPKB expression on PKB phosphorylation



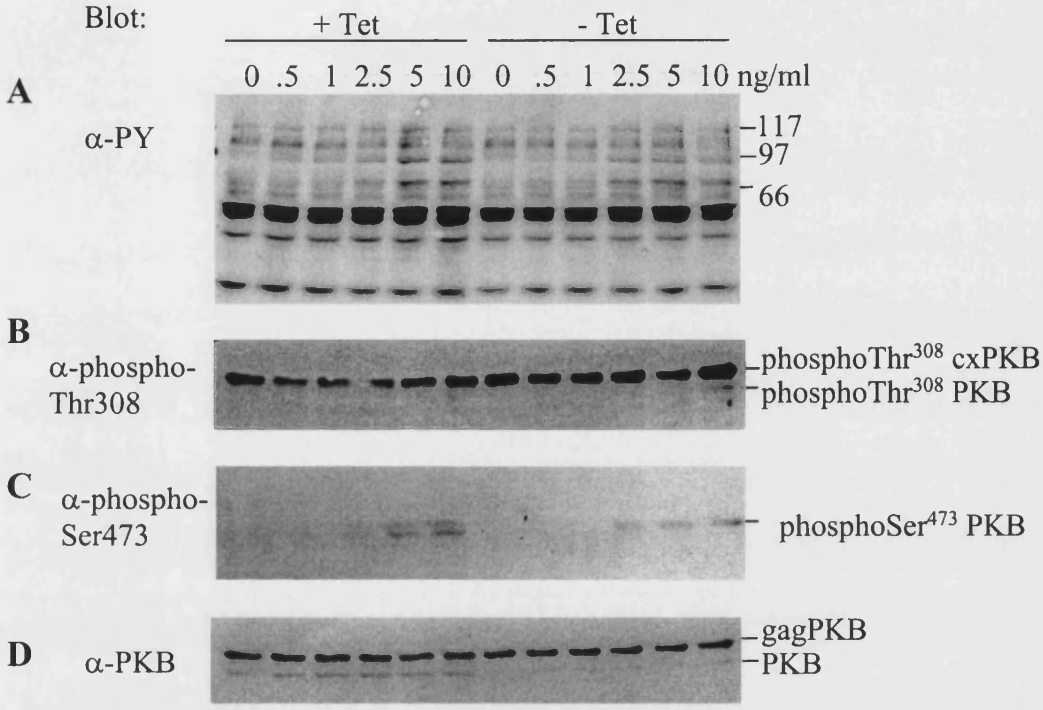
Cells were set up at $2x10^4$/ml in the absence (- Tet) or presence (+ Tet) of tetracycline for 24hr prior to starving of IL-3 and serum for 4hrs. Cells were stimulated for the indicated time with the 10ng/ml IL-3. Whole cell lysates ($4x10^5$ cell equivalents) were separated be SDS PAGE on duplicate gels and immunblotted with α- phospho-Ser^{473} (C) or α-phospho-Thr^{308} (B). Blots were stripped and reprobed with α-PKB (D), and α-phospho-tyrosine (A). This experiment is a representative of 2 experiments.

cxPKB has also been reported to act as a dominant negative and inhibit endogenous PKB activity. Therefore we would expect the phosphorylation of Ser^{473} and Thr^{308} to decrease due to cxPKB expression. The results shown in Figure 5.6 found very little endogenous PKB phosphorylation at either Ser^{473} or Thr^{308}. This is possibly due to the "leakiness" of the clone. In this experiment the expression of cxPKB is not very inducible and a high level of cxPKB expression is present even in the presence of tetracycline (Figure 5.13D). Therefore, if cxPKB is acting as a dominant negative, a low level of endogenous PKB phosphorylation would be expected. cxPKB is constitutively phosphorylated at Thr^{308} (Figure 5.6B). This is interesting and may account for cxPKB constitutive activity in *in vitro* kinase assays where the membrane is disrupted. However, no phosphorylation is detected at Ser^{473} (Figure 5.6C). The caax motif is attached to the C-terminal tail region of PKB where the Ser^{473} phosphorylation site is located and therefore may inhibit the access of PDK2 to this site.

The general leakiness of the 4H3 clone made it very difficult to determine what effects cxPKB was having on endogenous PKB activity in this system. However, work on the cxPKB clones has continued within the laboratory and using the 1E8 clone it has been found that cxPKB expression inhibits the phosphorylation of endogenous Ser^{473}, indicating that cxPKB is acting as a dominant negative (C. Beck and M.J. Welham, unpublished data).

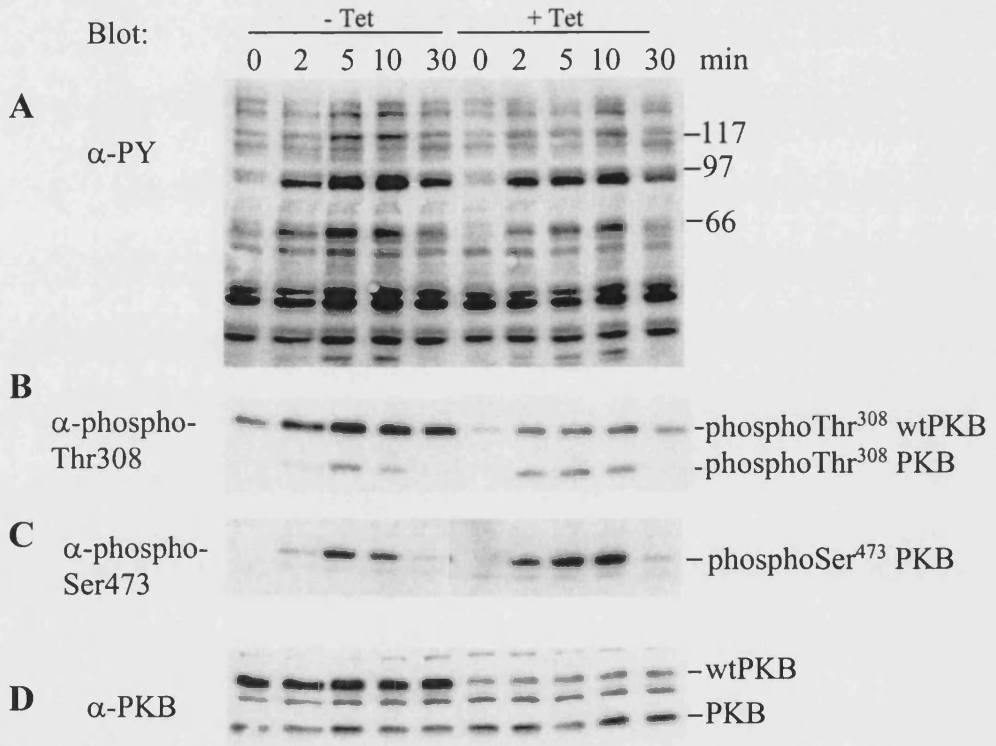
The wtPKB mutant was included in these studies primarily as a control and as such it was not expected to significantly affect endogenous PKB activity. However, as gagPKB and kdPKB appeared to be increasing the phosphorylation of endogenous PKB, it was possible that wtPKB may also have this effect. Surprisingly, preliminary analysis, have indicated that the expression of wtPKB decreases the phosphorylation of endogenous PKB at both Ser^{473} and Thr^{308} (Figure 5.7).

Figure 5.6
Effect of cxPKB expression on PKB phosphorylation



Cells were set up at $2x10^4$/ml in the absence (- Tet) or presence (+ Tet) of tetracycline for 24hr prior to starving of IL-3 and serum for 4hrs. Cells were stimulated for 5 minutes with the indicated concentration of IL-3. Whole cell lysates ($4x10^5$ cell equivalents) were separated be SDS PAGE on duplicate gels and immunblotted with α- phospho-Ser^{473} (C) or α-phospho-Thr^{308} (B). Blots were stripped and reprobed with α-PKB (D), α-phospho-tyrosine (A). This experiment is a representative of 2 independent experiments.

Figure 5.7
Effect of wtPKB expression on PKB phosphorylation



Cells were set up at $2x10^4$/ml in the absence (- Tet) or presence (+ Tet) of tetracycline for 24hr prior to starving of IL-3 and serum for 4hrs. Cells were stimulated with 10ng/ml IL3 for the indicated times. Whole cell lysates ($4x10^5$ cell equivalents) were separated be SDS PAGE on duplicate gels and immunblotted with α- phospho-Ser^{473} (C) or α-phospho-Thr^{308} (B). Blots were stripped and reprobed with α-PKB (D) and α-phospho-tyrosine (A). This experiment was performed once. Note that the order of samples has changed.

5.7 Effect of PKB Variant Expression on Bad Phosphorylation

The effects that these PKB variants have on the PKB signalling pathway is also dependent on their interactions with downstream effectors of PKB. For example, the kdPKB variant is reported to have no effect on endogenous PKB activation (and indeed in this system it appears to increase endogenous PKB activity), yet it acts as a dominant negative by competing with endogenous PKB for downstream targets. Therefore, to investigate the effects that our PKB variants are having on substrates of PKB we looked firstly at the hyper-phosphorylation of Bad. Bad is a pro-apoptotic member of the Bcl-2 family and when it is phosphorylated on serine residues its pro-apoptotic activity is abrogated. As previously discussed (Sections 3.15-3.18), hyper-phosphorylation of Bad results in a shift in its pattern of migration on SDS PAGE. In the previous experiments Bad was immuno-precipitated and run on 12.5% low bis gels to enhance the migration shift. However, in these experiments whole cells extracts were run on 20% gels and immuno-blotted with a different α-Bad antibody (R&D systems). The advantages to this method over the previous are that less sample is needed, an important consideration when cells must be seeded at a low concentration (10^5/ml) for optimal induction of expression, and using this method three phosphorylation states of Bad can be distinguished. The major disadvantage of examining Bad phosphorylation by looking at its shift in migration is that there is no way to distinguish which sites on PKB are being phosphorylated. PKB is known to phosphorylate Bad on Ser^{136}, but antibodies specific to the phosphorylated site were inadequate. Therefore this method was used to give an overall indication of the phosphorylation of Bad.

The expression of gagPKB, despite being constitutively active, does not dramatically increase the basal phosphorylation of Bad (Figure 5.8). Similarly the kinetics and levels of Bad phosphorylation in IL-3 induced cells are not significantly affected by the expression of gagPKB. It is interesting that at 30 and 60 minute time-points three phosphorylation species of Bad are distinguishable. The expression of kdPKB also had no detectable effect on the hyper-phosphorylation of Bad (Figure 5.9). However, the disadvantage of using gel shifts to investigate the PKB-catalysed phosphorylation of Bad is that it is unclear firstly which residues are being phosphorylated and therefore which kinases are responsible, and secondly, it is not known how many residues must be phosphorylated in order to detect a shift in migration. Therefore, these experiments do not conclusively determine what effects these PKB variants are having on substrates of PKB. Work on this continues within the laboratory and the point is illustrated by results using the cxPKB clone 1E8. Expression of cxPKB has no effect on the mobility

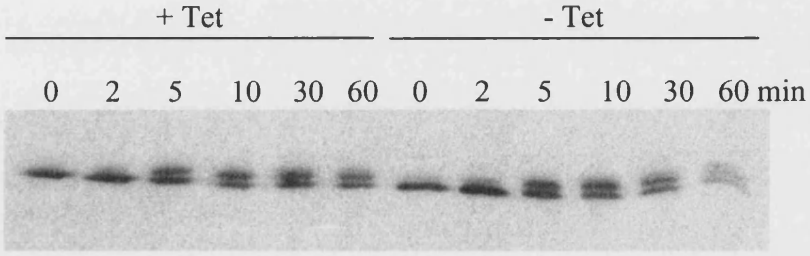
shift of Bad in response to IL-3 stimulation. However, cxPKB expression reduces the IL-3-induced phopshorylation of GSK3β (C. Beck and M.J. Welham, unpublished data).

5.8 Effects of PKB Variants on the IL-3-Induced Proliferation of BaF/3 Cells

The major functional effect of PI3K inhibition in BaF/3 cells is a dramatic reduction in the IL-3-induced proliferation (Figures 4.2 and 4.7, Craddock *et al.*, 1999). This correlates with the inhibition of PKB by both the PI3K inhibitor LY294002 (Figure 3.14) and Δp85 expression (Figures 4.5 and 4.6). PKB has previously been reported to be involved in proliferation in response to IL-2 stimulation (Brennan *et al.*, 1997; Ahmed *et al.*, 1997), but the expression of the constitutively active gagPKB in Δp85 expressing BaF/3 cells was not sufficient to rescue the proliferative defect (Figure 4.13). This suggested that PKB was not involved in the PI3K mediated proliferation of BaF/3 cells. However, a number of pathways in addition to PKB are mediated through PI3K and it is possible that inhibition of these pathways masked the effects of PKB on proliferation. Therefore, it was of interest to see what effects the expression of the PKB variants had on the IL-3-induced proliferation of BaF/3 cells. In these experiments, XTT assays were used to measure the metabolic activity of a population of cells and this reflects the level of proliferation of the cell population (Section 4.2).

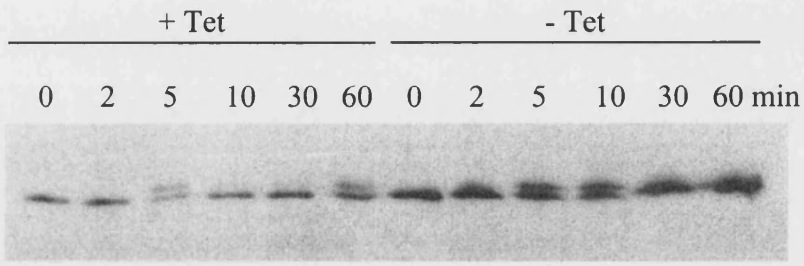
Ahmed *et al.* (1997) found that BaF/3 cells expressing a constitutively active, myristylated PKB mutant were more likely to be transformed and become factor-independent. If the expression of gagPKB was sufficient to make BaF/3 cells factor independent we would expect the cells to grow in the absence of IL-3 and the curve would shift to the left when gagPKB was expressed (-Tet). However, the removal of tetracycline did not induce a shift in the growth curve of gagPKB clones, with ED_{50} remaining at 0.6ng/ml (A) and 0.4ng/ml (B) in the presence and absence of tetracycline (Figure 5.10). Additionally, cells set up in media alone were dead after 72 hours in the presence or absence of tetracycline, indicating that the expression of gagPKB was not sufficient to transform BaF/3 cells. These data are supported by previous work where gagPKB was unable to restore the Δp85-inhibited proliferation in this cell line and did not make them factor independent (Figure 4.13).

Figure 5.8
Effect of gagPKB expression on Bad phosphorylation



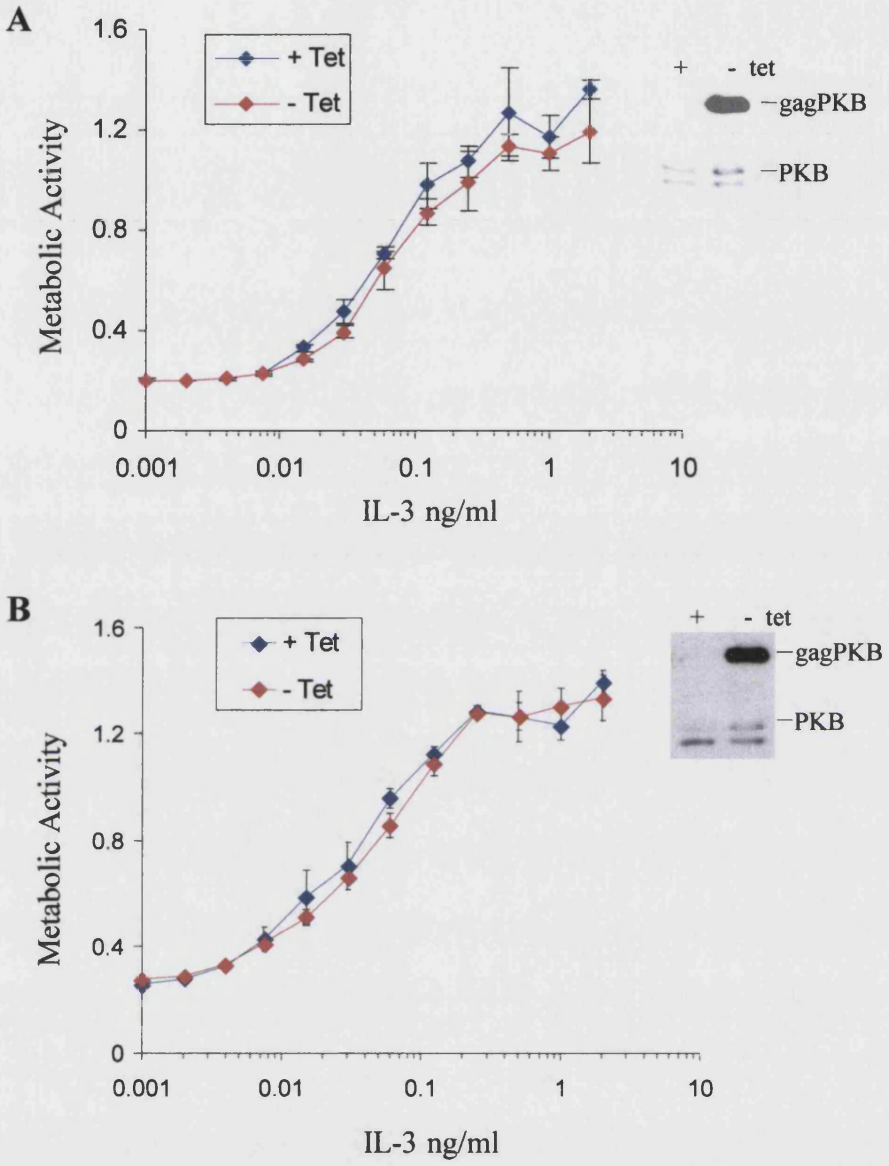
Cells (clone 1C10) were set up at 10^5/ml in the presence (+ Tet) or absence (- Tet) of tetracycline for 24 hrs prior to starving of IL-3 and serum for 4 hrs. Cells were stimulated with 10ng/ml IL-3 for the indicated times. Whole cell lysates ($4x10^5$ cell equivalents) were separated on 20% SDS PAGE gels and immunoblotted with α-Bad (R&D).. This experiment was performed once.

Figure 5.9
Effect of kdPKB expression on Bad phosphorylation



Cells (clone D12) were set up at 10^5/ml in the presence (+ Tet) or absence (- Tet) of tetracycline for 24 hrs prior to starving of IL-3 and serum for 4 hrs. Cells were stimulated with 10ng/ml IL-3 for the indicated times. Whole cell lysates ($4x10^5$ cell equivalents) were separated on 20% SDS PAGE gels and immunoblotted with α-Bad (R&D). This experiment was performed once.

Figure 5.10
Effect of gagPKB Expression on IL-3 induced Proliferation of BaF/3 Cells



BaF/3 cells expressing gagPKB, **(A)** clone 1C10 and **(B)** clone 1F9, were washed free of factor and tetracycline and set up in the presence (+) or absence (-) of tetracycline with IL-3 (at a starting concentration of 2ng/ml with serial 1:2 dilutions across a 96 well plate), or serum alone as described in materials and methods. After 72 hours the assays were developed. The mean values (n=3) with standard deviation are plotted. **Insert**: At the same time that the XTT assays were set up, cells were grown +/- tetracycline for 72 hours. Cell extracts were separated by SDS PAGE and immunoblotted with α-PKB to see protein expression. This experiment is representative of 3 independent experiments and similar results were found with one other clone.

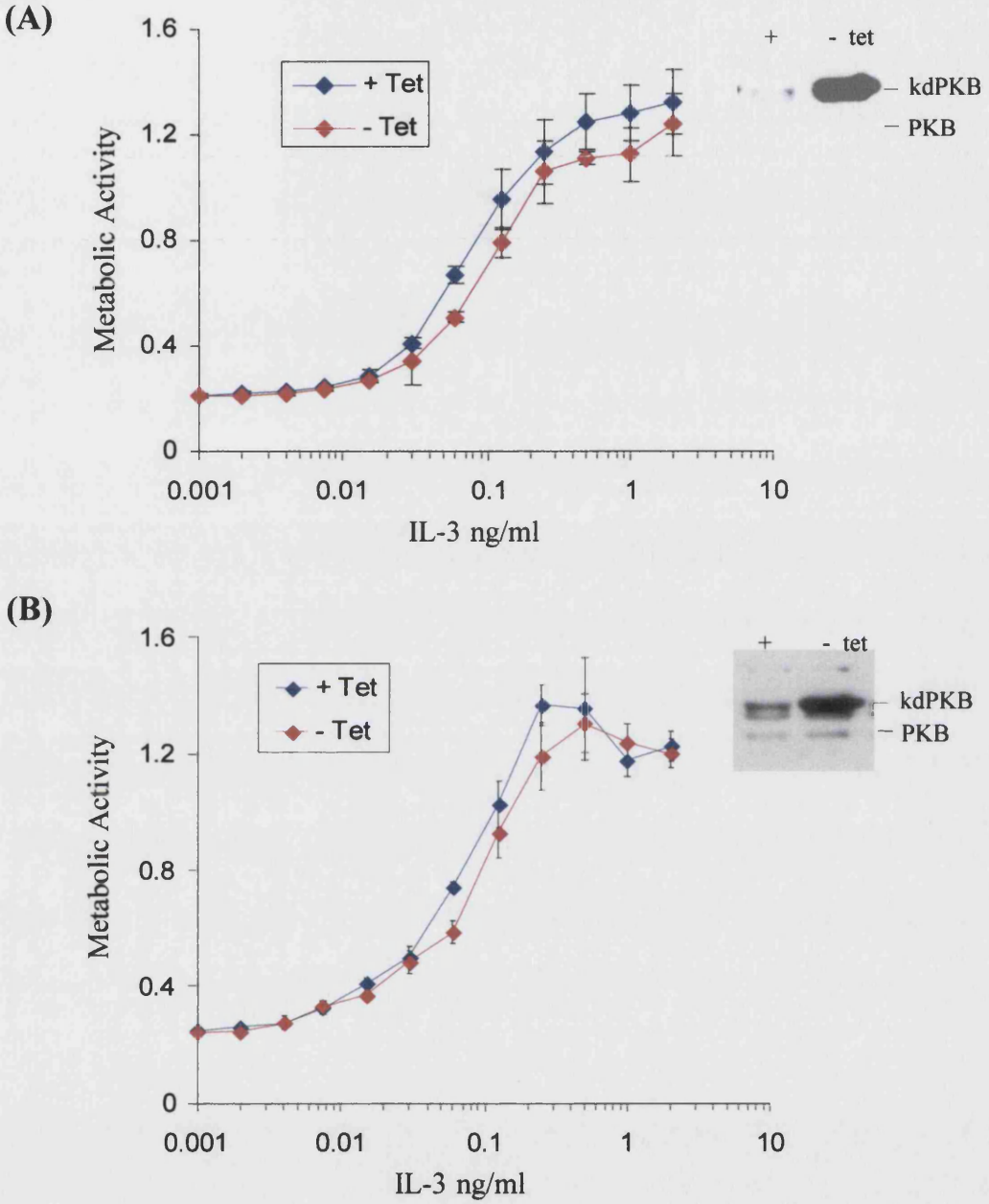
It is unclear from previous experiments whether kdPKB is acting as a dominant negative or not in these cells. Nevertheless, the effects of kdPKB expression were investigated in the IL-3-driven proliferation of BaF/3 cells. The IL-3 driven proliferation of BaF/3 cells was not affected by the expression of kdPKB (Figure 5.11). The ID_{50} values were slightly higher than seen in the gagPKB clones but were not significantly altered by the withdrawal of tetracycline.

cxPKB, despite the leakiness of the 4H3 clone, appears to be inhibiting the IL-3 induced phosphorylation of PKB (C. Beck and M.J. Welham, unpublished). Therefore, if PKB is involved in the proliferation of BaF/3 cells, the expression of cxPKB clones would inhibit the IL-3-induced proliferation. However, the removal of tetracycline had no significant effect on the IL-3-induced proliferation of cxPKB clones (Figure 5.12). When the inducibility of cxPKB expression was checked a high level of expression was observed in the presence of tetracycline (Figure 5.12, insert). Therefore, it is difficult to determine from this result what the effects of cxPKB expression on proliferation are. However, XTT assays performed with the 1E8 clone, which is tightly regulated by tetracycline, indicate that cxPKB expression does not affect the IL-3-induced proliferation of BaF/3 cells (C. Beck and M.J. Welham, unpublished data)

The wtPKB mutant is identical to cellular PKB with the exception of the myc tag. Therefore, we would expect that expressing wtPKB would enhance any functional effects of PKB. As such, if PKB is involved in the IL-3-induced proliferation of BaF/3 cells, we would expect wtPKB to enhance this proliferation. However, the expression of wtPKB did not enhance the IL-3-induced proliferation (Figure 5.13).

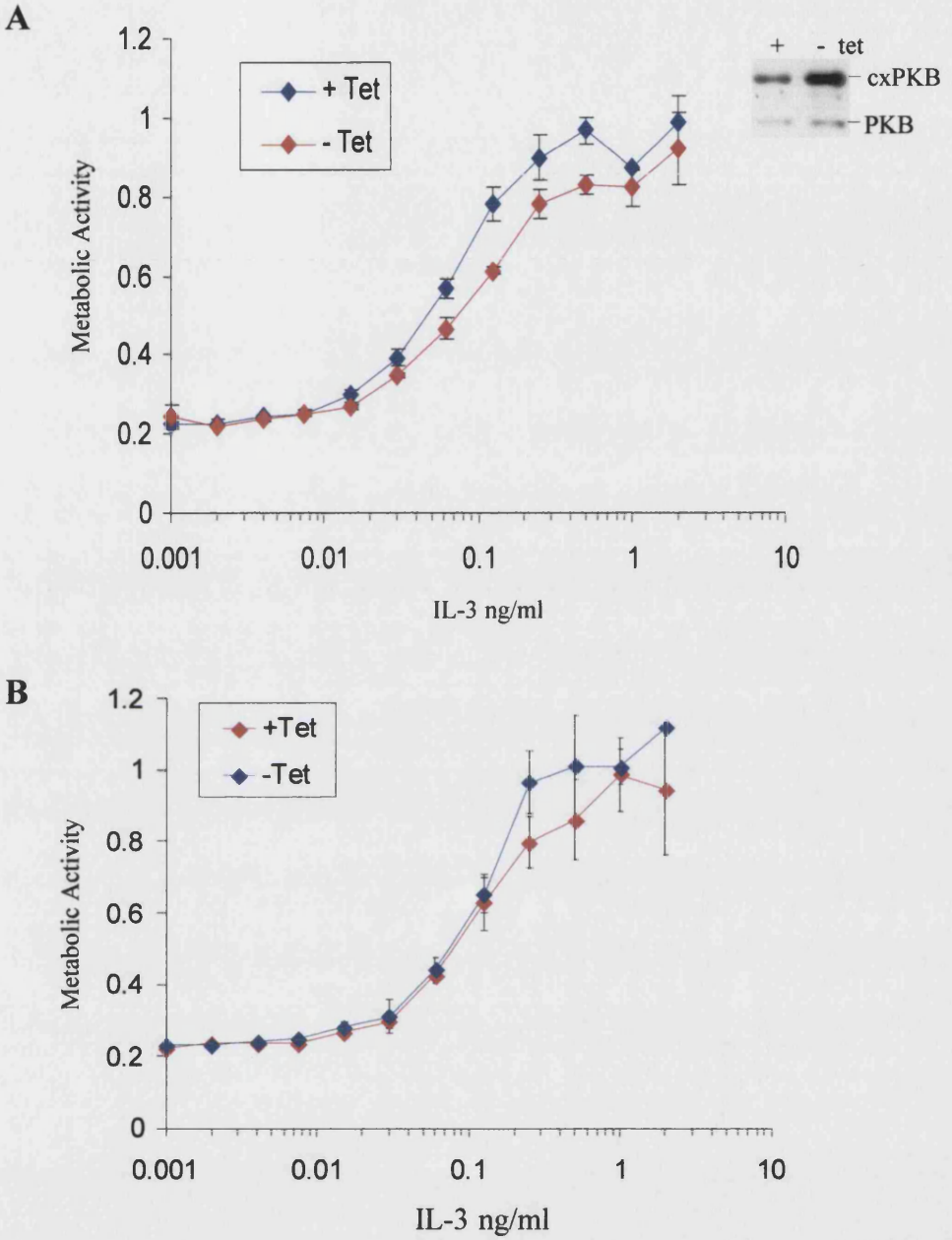
The data from these XTT assays show that the expression of a constitutively active PKB mutant (gagPKB) was not able to negate the dependence of BaF/3 cells for IL-3 for proliferation nor was the expression of a dominant negative PKB (cxPKB) able to inhibit the IL-3- induced proliferation. Additionally, the kdPKB and wtPKB variants, which increase the activation of endogenous PKB, did not affect the IL-3-induced proliferation. Taken together, these data suggest that PKB is not involved in the IL-3-driven proliferation of these cells.

Figure 5.11
Effect of kdPKB Expression on IL-3 induced Proliferation of BaF/3 Cells



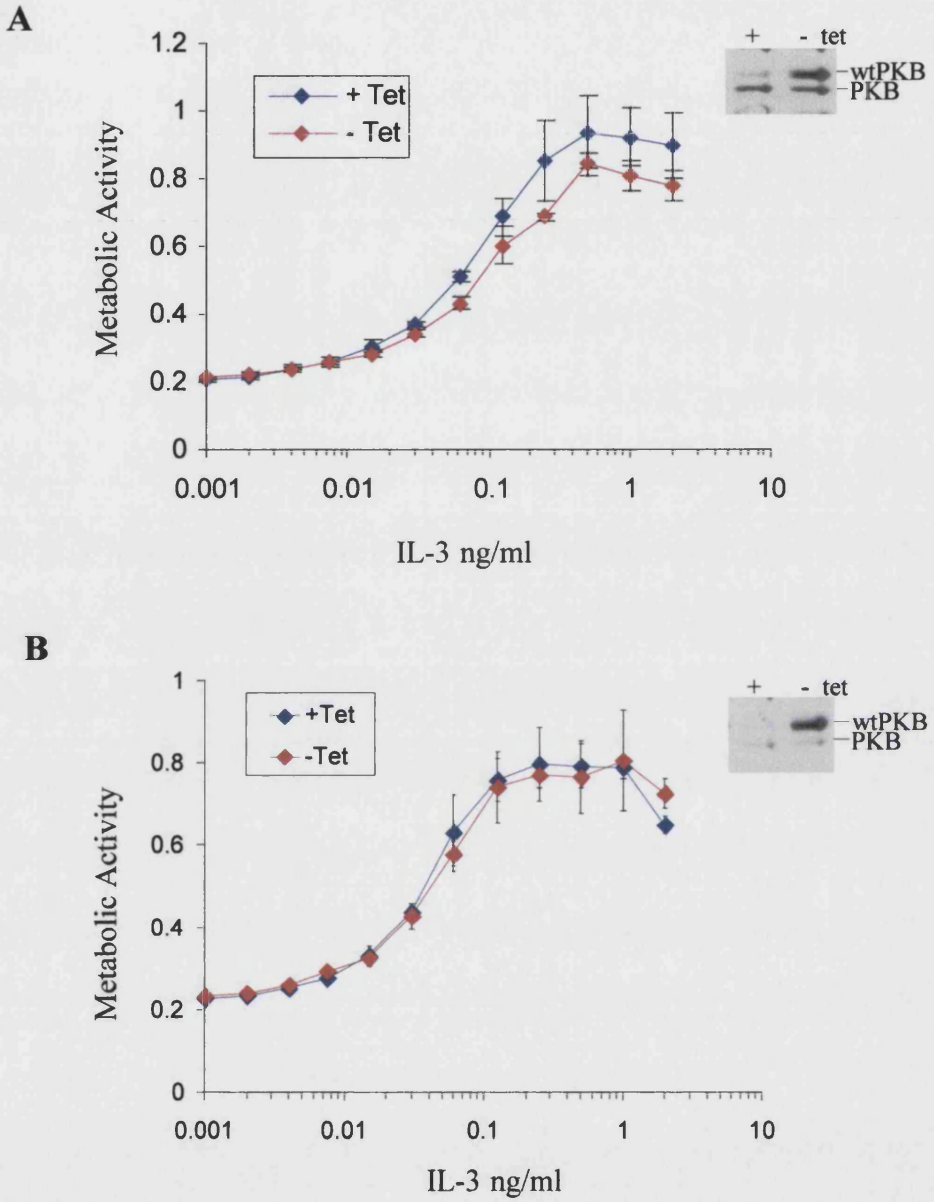
BaF/3 cells expressing kdPKB, **(A)** clone D12 and **(B)** clone A9, were washed free of factor and tetracycline and set up in the presence (+) or absence (-) of tetracycline with IL-3 (at a starting concentration of 2ng/ml with serial 1:2 dilutions across a 96 well plate), or serum alone as described in materials and methods. After 72 hours the assays were developed. The mean values (n=3) with standard deviation are plotted. **Insert**: At the same time that the XTT assays were set up, cells were grown +/- tetracycline for 72 hours. Cell extracts were separated by SDS PAGE and immunoblotted with α-PKB to see protein expression. This experiment is representative of 3 independent experiments and similar results were found with 3 different clones.

Figure 5.12
Effect of cxPKB Expression on IL-3 induced Proliferation of BaF/3 Cells



BaF/3 cells expressing cxPKB, **(A)** clone 4H3 and **(B)** clone 2F5 were washed free of factor and tetracycline and set up in triplicate in the presence (+) or absence (-) of tetracycline with IL-3 (at a starting concentration of 2ng/ml with serial 1:2 dilutions across a 96 well plate), or serum alone as described in materials and methods. After 72 hours the assays were developed. The mean values (n=3) with standard deviation are plotted. **Insert** At the same time that the XTT assays were set up, cells were grown +/- tetracycline for 72 hours. Cell extracts were separated by SDS PAGE and immunoblotted with α-PKB to see protein expression. This experiment is representative of 3 independent experiments and was performed with 3 different clones.

Figure 5.13
Effect of wtPKB Expression on IL-3 induced Proliferation of BaF/3 Cells



BaF/3 cells expressing wtPKB, **(A)** clone 3G10 and **(B)** clone 3G4,were washed free of factor and tetracycline and set up in triplicate in the presence (+) or absence (-) of tetracycline with IL-3 (at a starting concentration of 2ng/ml with serial 1:2 dilutions across a 96 well plate), or serum alone as described in materials and methods. After 72 hours the assays were developed. The mean values (n=3) with standard deviation are plotted. **Insert:** At the same time that the XTT assays were set up, cells were grown +/- tetracycline for 72 hours. Cell extracts were separated by SDS PAGE and immunoblotted with α-PKB to see protein expression. This experiment is representative of 3 independent experiments and similar results were obtained with 3 different clones.

5.9 Effects of PKB Variants on the Viability of BaF/3 Cells

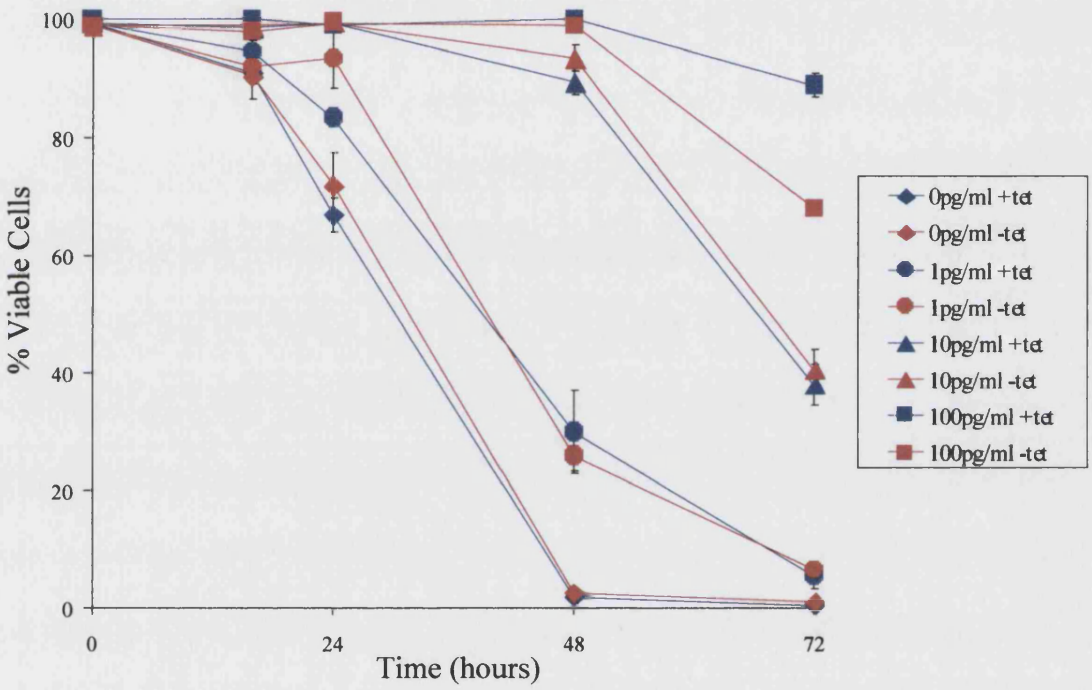
Over recent years PKB has been implicated in the survival of an increasing number of cell types in response to an expansive array of stimuli (Reviewed in Coffer and Woodgett, 1998, Datta *et al.*, 1999, Vanhaesebroeck and Alessi, 2000). However, the results presented in Chapters 3 and 4 question the generally accepted dogma that the activation of PKB is both sufficient and necessary for cell survival. In order to investigate this more thoroughly, we looked at the viability of the PKB clones in the presence and absence of tetracycline. Cells were grown over a period of 3 days in limiting concentrations of IL-3, or in the absence of IL-3, and the percentage of viable cells were determined at specific time intervals.

The expression of the constitutively active PKB variant, gagPKB, did not afford any extra protection from death to those cells grown in limiting amounts of IL-3 (Figure 5.14). The survival of cells grown at 100pg/ml IL-3 appears to decrease in the absence of tetracycline at 72 hours. However, at this time point the cells are beginning to die as a result of overcrowding and this should be kept in mind when interpreting this result. Similarly, the expression of the kdPKB mutant did not provide these cells with any increased or decreased protection from death (Figure 5.15).

If PKB could provide a survival signal to BaF/3 cells, we would expect that expression of the dominant negative cxPKB would increase cell death at low IL-3 concentrations. However, the expression of cxPKB did not increase death when cells were grown in 50pg/ml IL-3 or in the absence of IL-3 Figure 5.16). A small decrease in cell death (10%) was observed in cxPKB expressing cells grown in 1pg/ml IL-3. However, the significance of this result is not clear, as it was not observed when the experiment was repeated nor in the 1E8 or 2G6 clones (C. Beck and M.J. Welham, unpublished).

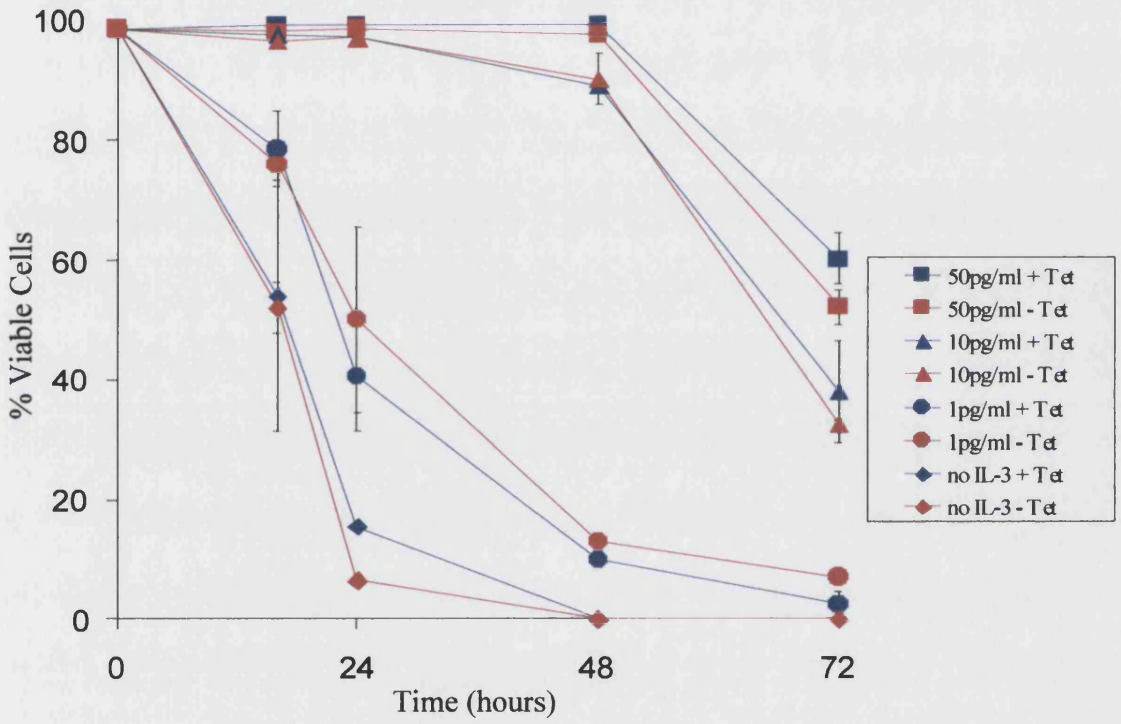
These results demonstrate that expression of gagPKB in BaF/3 cells is unable to protect cells from death in the absence of IL-3. This suggests that PKB alone is not sufficient to provide a growth signal. Additionally, kdPKB and cxPKB did not increase cell death, which poses the question of whether PKB is involved in the survival of all cell types in response to all cytokines.

Figure 5.14
The effect of gagPKB expression on the viability of BaF/3 cells



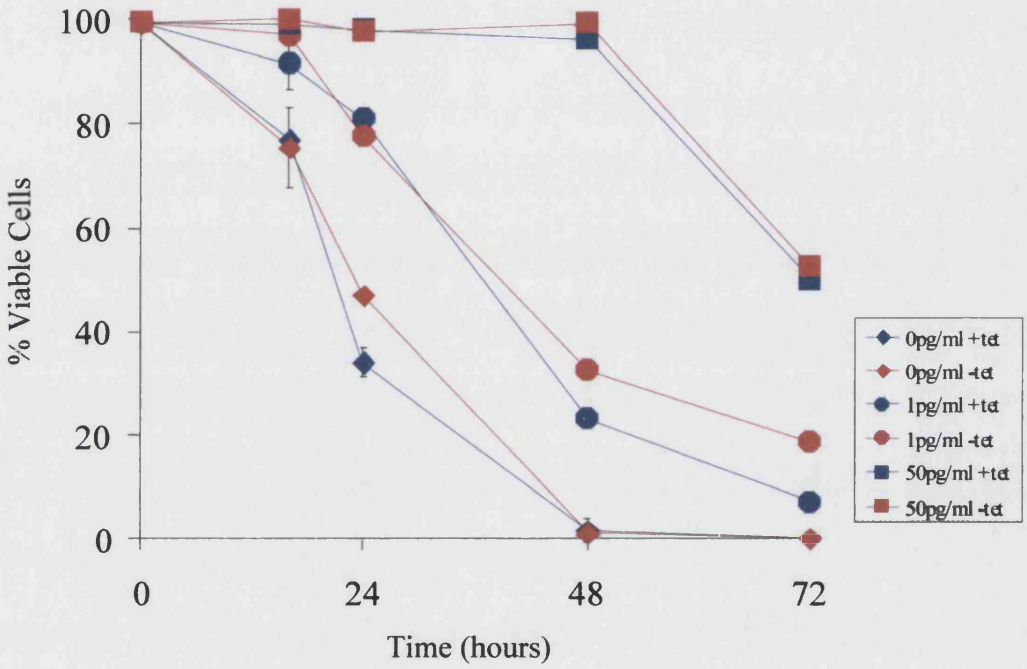
BaF/3 cells expressing gagPKB were set up in the presence (+ Tet) or absence (- Tet) of tetracycline. After 24 hours the two populations of cells were washed free of factor and grown in the varying concentrations of IL-3. The percentage of live cells at the indicated times were determined by trypan blue exclusion assays. The mean values (n=2) are plotted with standard deviation. This experiment was performed once.

Figure 5.15
The effect of kdPKB expression on the viability of BaF/3 cells



BaF/3 cells expressing kdPKB were set up in the presence (+ Tet) or absence (- Tet) of tetracycline. After 24 hours the two populations of cells were washed free of factor and grown in the varying concentrations of IL-3. The percentage of live cells at the indicated times were determined by trypan blue exclusion assays. The mean values (n=2) are plotted with standard deviation. This experiment was performed twice.

Figure 5.16
The effect of cxPKB expression on the viability of BaF/3 cells



BaF/3 cells expressing cxPKB were set up in the presence (+ Tet) or absence (- Tet) of tetracycline. After 24 hours the two populations of cells were washed free of factor and grown in the varying concentrations of IL-3. The percentage of live cells at the indicated times were determined by trypan blue exclusion assays. The mean values (n=2) are plotted with standard deviation. This experiment was performed twice.

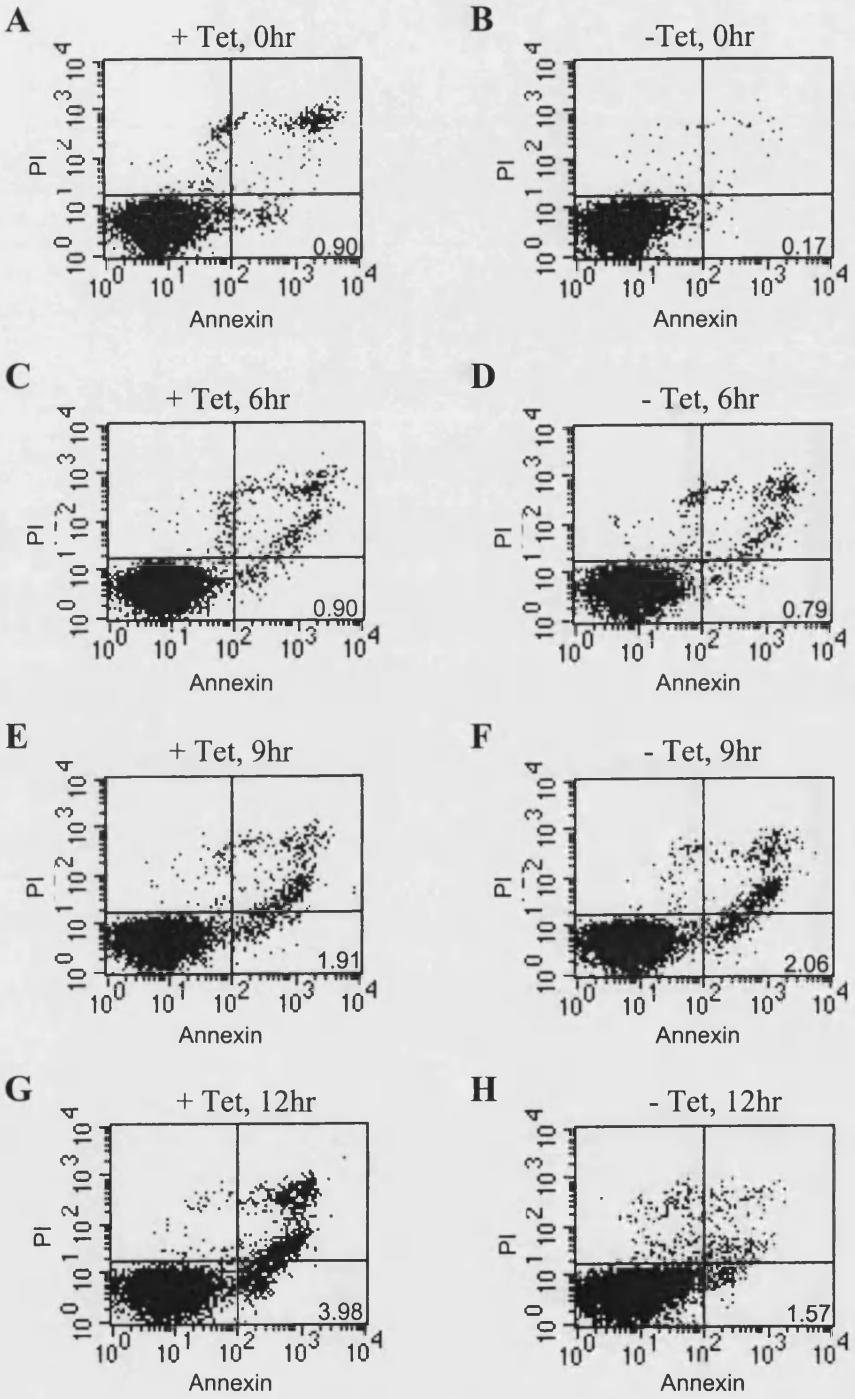
5.10 Effect of PKB Variants on Apoptosis

The viability assays shown previously (Figures 5.14-5.16) examined the effect of PKB variants on the survival of BaF/3 cells. The expression of a constitutively active PKB (gagPKB) did not protect the cells from death induced by IL-3 withdrawal, nor did the expression of a dominant negative PKB (cxPKB) increase cell death. BaF/3 cells are dependent on IL-3 for their continued growth and survival and will by die by apoptosis upon IL-3 withdrawal. Therefore, the role of PKB in the survival of BaF/3 cells was further examined by looking at the rate of apoptosis of the PKB variants. Apoptosis was measured by FACS analysis of cells stained with propidium iodide and annexinV.

Ahmed *et al.* (1997) reported that the expression of myristylated PKB in BaF/3 cells was sufficient to transform these cells, preventing their death upon IL-3 withdrawal. However, the constitutively active gagPKB mutant was unable to promote survival in the absence of IL-3 (Figure 5.14). To investigate this in more depth, we looked at the apoptosis of the gagPKB clone 1C10 in the absence of IL-3 and in conditions of limited IL-3.

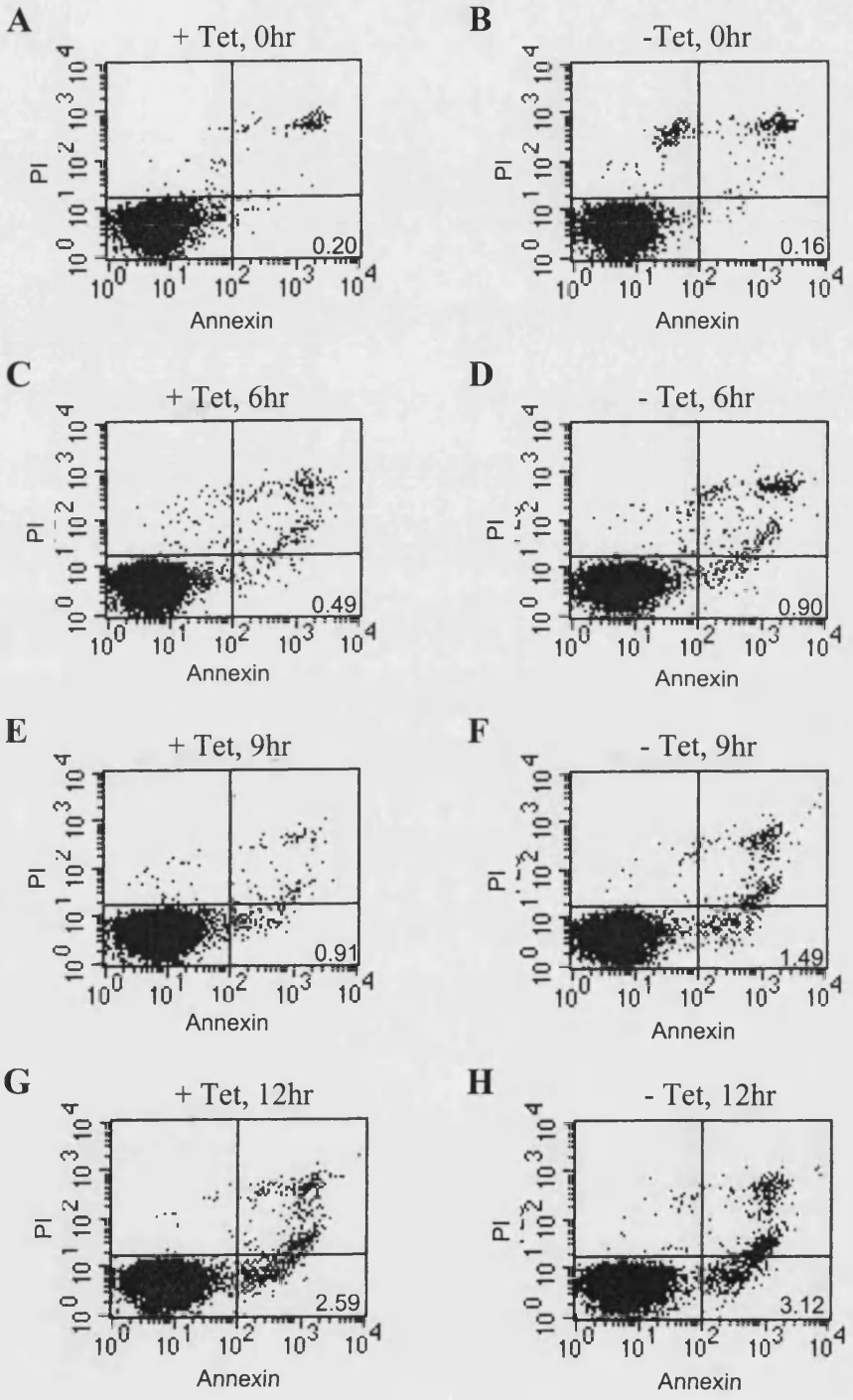
First the rate of apoptosis in the presence and absence of tetracycline was examined in BaF/3 cells that had been transfected with "empty" pUHD10-5 vector and the regulatory pUHD15-1 vector. The dot plots shown in Figure 5.17 follow the apoptosis of these cells over twelve hours after IL-3 withdrawal. The lower right quadrant contains cells that are stained with annexinV alone, which is used to identify those cells undergoing apoptosis as opposed to necrotic cell death. This Figure illustrates the progression of unstained live cells (lower left quandrants) to those cells that are in the early stages of apoptotic cell death (lower right quandrants) and finally to their death (upper quadrants). The progression of apoptosis induced by IL-3 withdrawal of gagPKB cells is shown in Figure 5.18. The number of cells undergoing apoptosis at each time point does not vary significantly in the absence or presence of tetracycline, indicating that gagPKB expression is not protecting the cells from apoptosis in the absence of IL-3. The apoptotic profile observed with the gagPKB clone is similar to that seen in BaF/3 cells transfected with an "empty" vector (Figure 5.17). The apoptosis of gagPKB cells was also investigated for up to 24 hours after IL-3 deprivation and at low concentration of IL-3 (1pg/ml and 10pg/ml). However, the expression of gagPKB did not afford any protection from apoptosis in these experiments.

Figure 5.17
Effect of Empty Vector Expression on Apoptosis



Empty vector clone was induced for 24 hours in the presence or absence of tetracycline, prior to being washed free of IL-3 and seeded at 10^4/ml. After the indicated time cells were washed and stained with AnnexinV and propidium iodide. Cells were analysed by flow cytometry and 10 000 events were recorded.

Figure 5.18
Effect of gagPKB expression on Apoptosis

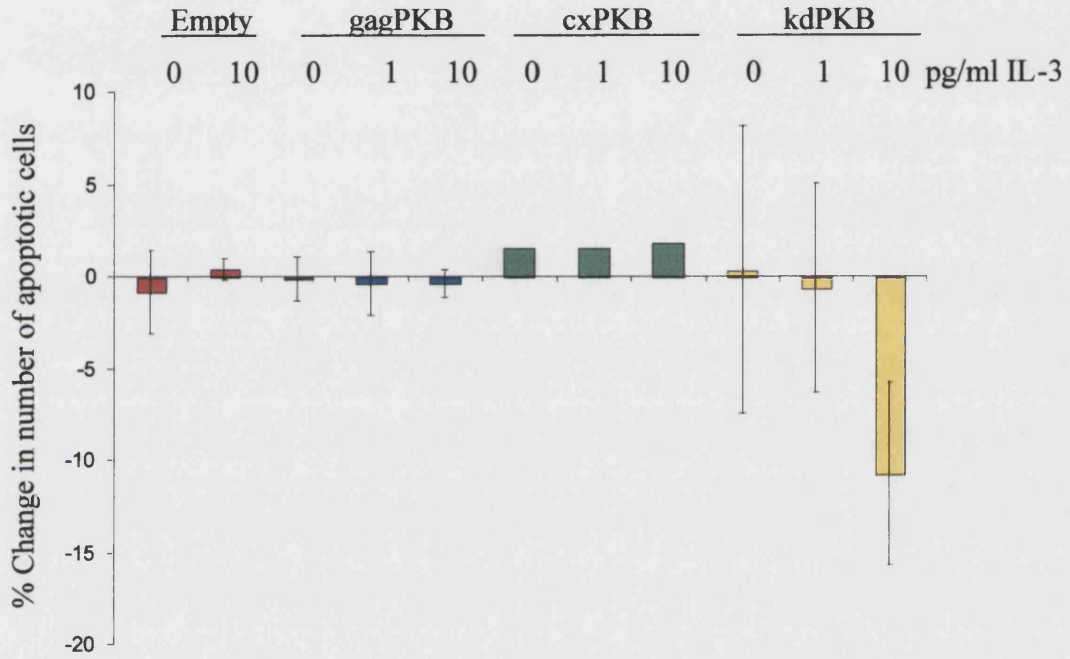


gagPKB clone 1C10 was induced for 24 hours in the presence or absence of tetracycline, prior to being washed free of IL-3 and seeded at 10^4/ml. After the indicated time cells were washed and stained with AnnexinV and propidium iodide. Cells were analysed by flow cytometry and 10 000 events were recorded.

To compare the effects of the expression of these PKB variants on apoptosis, a 12 hour time-point was chosen. Figure 5.18 compares the change in the number of cells undergoing apoptosis in the presence of tetracycline (no expression) to in the absence of tetracycline (expression of PKB variant). Therefore, negative values denote an decrease in the number of cells undergoing apoptosis upon expression of the PKB variant. gagPKB did not protect BaF/3 cells from apoptosis even in low concentration of IL-3. cxPKB, perhaps increased the number of apoptotic cells by a small amount but it must be remembered that this was only one experiment. kdPKB gave widely varying results between experiments and it is difficult to determine what its effects on apoptosis are. However, the results obtained with the expression of the kdPKB variant do illustrate the shortcomings of such a comparison. In this experiment we are measuring the difference in apoptotic cell number in cells grown in limited IL-3 in the presence or absence of tetracycline. The expression of exogenous PKB is induced by the withdrawal of tetracycline for 24 hours before the cells are washed and seeded with low IL-3. Therefore, we are comparing the number of apoptotic cells in two distinct cell populations, which may have differed in apoptotic cell number at the beginning of the experiment.

These concerns aside, the results presented above suggest that the expression of these PKB variants does not result in either a massive increase or decrease in apoptosis. Taken together, with the results presented in previous chapters, they suggest that PKB is not ultimately required for the survival of all cell types and question whether indeed it is involved in the survival of all cells.

Figure 5.19
Change in Apoptotic Cell Number Induced by PKB Variant Expression



Cells were set up at 10^5/ml in the absence or presence of tetracycline for 24 hours prior to being cultured in the indicated concentration of IL-3. 12-14 hours later, cells were washed, stained with AnnexinV and propidium iodide and the number of apoptotic cells determined by FACS analysis. The values indicated here, give the increase or decrease in the number of cells undergoing apoptosis when the PKB variant was expressed. Thus, positive values indicate an increase in apoptosis upon PKB variant expression, and negative value indicate a decrease in the number of cells undergoing apoptosis when the PKB variant is expressed. The results shown are +/- standard deviation and the experiments were repeated (empty) 2, (gagPKB) 4, (cxPKB)1 and (kdPKB) 2 times.

5.11 Discussion

The serine threonine kinase PKB has been implicated in the growth and survival of many cell systems and in response to a number of stimuli (Reviewed in Coffer and Woodgett,. 1998; Datta *et al.*, 1999; Vanhaesebroeck and Alessi, 2000). This led to the general belief, that the activation of PKB was both sufficient and necessary for the generation of a survival signal. However, an absolute correlation between PKB activation, Bad phosphorylation and cell survival and proliferation was not found (Chapters 3 and 4), which raised the question of how important and necessary PKB activation is for these events. In these experiments, PI3K inactivation was used to inhibit the activity of PKB, but the effects of direct PKB inhibition were not examined. To enable the effects of PKB on IL-3 signalling to be examined directly, PKB variants were expressed in BaF/3 cells. These created cell models in which PKB activity was either inhibited or less dependent of IL-3 stimulation.

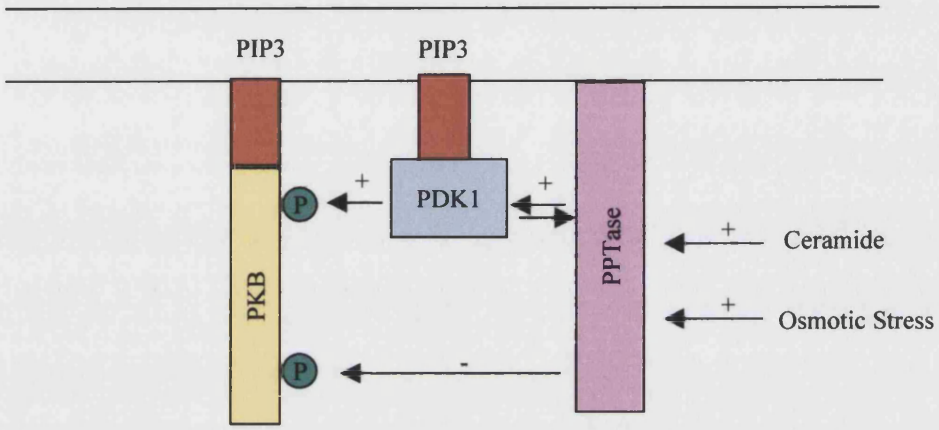
Two PKB variants that were reported to inhibit PKB signalling were transfected into BaF/3 cells. The first, kdPKB is catalytically inactive, however expression of this mutant in BaF/3 cells resulted in an increase in the phosphorylation of endogenous PKB in response to IL-3 stimulation. Phosphorylation at Ser^{473} correlates with the activity of PKB (Figures 3.1-3.5) and this was used to investigate the activity of endogenous PKB, as opposed to exogenous PKB activity. This was a surprising result especially given that the expression of kdPKB in other systems did not result in an increase in endogenous PKB activity (Kotani *et al.*, 1999; Takata *et al.*, 1999) or co-expressed wtPKB activity (van Weeren *et al.*, 1998). *In vitro* kinase assays of kdPKB expressing clones pre-cleared with 9E10 antibody could be performed to confirm that the activity of endogenous PKB activity is increased by kdPKB expression. kdPKB is believed to act as a dominant negative in some systems by competing with endogenous PKB for its downstream effectors (Dudek *et al.*, 1997; Datta *et al.*, 1997; del Peso *et al.*, 1997; Kulik and Weber, 1998). However, its expression in BaF/3 cells did not interfere with the hyper-phosphorylation of Bad induced by IL-3 stimulation (Figure 5.9). This is in contrast to 3T3 cells where kdPKB expression inhibited the shift in migration that is indicative of Bad hyper-phosphorylation (Datta *et al.*, 1997). However, van Weeren *et* al. (1998) also report that the dominant negative effects of this PKB mutant are variable.

The second putative dominant negative PKB used in these studies was the cxPKB. *In vitro* kinase assays of this mutant had a constitutive level of activity, which was not increase by stimulation with IL-3. Van Weeren *et al.* (1998) also examined the activity of cxPKB. They found that the level of cxPKB activity in both unstimulated and insulin stimulated A14 cells was comparable to that of unstimulated wtPKB. However, considerably less cxPKB than wtPKB was expressed in their cells making it difficult to determine whether cxPKB also has a low level of constitutive activity in this cell line. When the phosphorylation at Ser^{473} and Thr^{308} of cxPKB was examined, it was constitutively phosphorylated on Thr^{308} (Figure 5.6). As phosphorylation at this site contributes to activation (Alessi *et al.*, 1996) this could account for the basal level of cxPKB activity observed. The effect of cxPKB expression on the activation of endogenous PKB was more difficult to determine due to the constitutive expression of the 4H3 clone. Results in the 1E8 clone suggest that the expression of cxPKB decreases the phosphorylation of endogenous PKB (C. Beck and M.J. Welham, unpublished) and low levels of endogenous phosphorylation were observed in 4H3 clone, which could be a result of the dominant negative effects of cxPKB (Figure 5.3). However, given the low constitutive activity of cxPKB (whether or not this is an artifact of the assay system) the effects of cxPKB expression on downstream targets of PKB need to be examined. Further work within the laboratory has found that the expression of cxPKB resulted in a small reduction of GSK3β phosphorylation, but did not inhibit the IL-3-induced hyper-phosphorylation of Bad or affect Bad Ser^{112} phosphorylation (C. Beck and M.J. Welham, unpublished data).

Other dominant negative PKB variants have since been reported. Of particular interest is the AAA mutant, which contains 3 point mutations of Lys^{179}Ala, Thr^{308}Ala and Ser^{473}Ala. This PKB variant as well as being catalytically inactive inhibits the activity of co-transfected wtPKB (Wang *et al.*, 1999), which suggests that it may be more efficient in its dominant negative effects. It would be interesting to examine its effects in BaF/3 cells.

The constitutively active PKB variant gagPKB was chosen to provide an active PKB signal that is independent of IL-3 activity. This variant was found to have a low constitutive activity, which was increased upon IL-3 stimulation. Others have also found the activity of the gagPKB mutant to increase upon stimulation (Burgering and Coffer, 1995). Given the high levels of basal phosphorylation of gagPKB in Δp85 expressing cells (Figure 4.11 and 4.12), it was surprising that a similar high level of basal phosphorylation was not observed in gagPKB cells (Figure 5.4). While, the low level of gagPKB expression in this experiment may be a reason for the disparity in phosphorylation levels observed, it is interesting that in PDK-1 -/- ES cells a similar high level of Ser^{473} phosphorylation was also present in unstimulated cells (Williams *et al.*, 2000). As this effect was abolished by wortmannin treatment, it was explained by the high basal level of PI3K activity that was observed in these cells. However, it could also so be due to a decreased dephosphorylation of Ser^{473}. When PKB was targeted to the membrane it was activated by phosphoryalation at Ser^{473} and Thr^{308}. When PI3K was inactivated by wortmannin, LY294002 or Δp85, PKB was presumably not targeted to the membrane and PKB was not phosphoryated at either site (Williams *et al.*, 2000; Figures 3.11-3.14, 4.5 and 4.6). However, when PKB was targeted to the membrane in the absence of PI3K activity (Figures 4.11 and 4.12) the increase in phosphorylation was much greater than that seen in presence of PI3K activity (Figure 5.4). This effect was more pronounced when looking at Ser^{473} phosphorylation. A possible explanation for this is that the phospho-lipid products of PI3K, PIP_3, also target PDK1 to the membrane. Therefore, a membrane targeted PDK-1 may be required for the dephosphorylation of PKB at Ser^{473}. Thus, in the PDK-1 -/- cells where there is no PDK-1, phopshorylation of PKB at Ser^{473} is enhanced. Therefore, PDK-1 recruitment to the membrane by PIP_3 may activate a phosphatase activity, which is active on Ser^{473}. This could act as a negative feed back loop by which the positive regulatory signals of PKB are switched off (Figure 5.20).

Figure 5.20
Possible feedback loop of PKB activation



Putative feedback loop involved in the regulation of PKB. PKB is activated by a combination of membrane targeting through the PH domain (■) and phosphorylation on Ser^{473} and Thr^{308}. Thr^{308} is phosphorylated by PDK1 through a mechanism which is dependent on the presence of the phospholipid products of PI3K. When PKB is membrane targeted in the absence of phospholipids or PDK1 phosphorylation at Ser^{473} is increased suggesting the PDK1 or phospholipid regulated dephosphorylation of PKB at Ser^{473}. Ceramide and osmotic stress activate a phosphatase activity which may catalyse the dephosphorylation of PKB at Ser^{473}.

Recent work has identified a pathway that leads to the dephosphorylation of PKB on Ser^{473} (Schubert *et al.*, 2000; Zinda *et al.*, 2001; Hajduch *et al.*, 2001) and Thr^{308} (Zinda *et al.*, 2001; Hajduch *et al.*, 2001). A number of groups had reported that PKB activity is regulated in part by the membrane sphingolipid, ceramide and that this regulation is independent of PI3K-catalysed phospholipid production (Zhou *et al.*, 1998; Schubert *et al.*, 2000; Zinda *et al.*, 2001; Hajduch *et al.*, 2001). Ceramide production results in the activation of various proteins including the protein phosphatase CAPP (ceramide activated protein phosphatase) (Dobrowsky and Hannun, 1992; Peterson and Schreiber, 1999). The ceramide-induced inhibition of PKB phosphorylation is sensitive to the phosphatase inhibitors okadaic acid and calyculin, which has lead to the speculation that CAPP may catalyse the dephosphorylation of PKB (Schubert *et al.*, 2000; Zinda *et al.*, 2001; Hajduch *et al.*, 2001). Schubert et al. (2000) who were investigating GM-CSF-stimulated TF1 cells, found that only phosphorylation at Ser^{473} was affected by ceramide treatment. Surprisingly, when these cells were treated with the phosphatase inhibitors okadaic acid and calyculin, phosphorylation of PKB at Thr^{308} was completely abolished. This suggests that a dephosphoryation event is involved in the PDK-1-catalysed phosphorylation of Thr^{308}, which is surprising given the constitutive activity of PDK-1. These findings demonstrate that the regulation of PDK-1 is more complicated than at first thought and point towards the involvement of a phosphatase. Given these findings it would be interesting to investigate the role of phosphatases in the phosphorylation of Thr^{308} by PDK-1 and the possible role of PDK-1 in the dephosphorylation of Ser^{473} by a phosphatase, perhaps CAPP.

It has been reported that osmotic stress can stimulate the production of $PI(3,4,5)P_3$ without inducing the activation of PKB and indeed osmotic stress was shown to inhibit the IGF-1-induced activation of PKB (Van der Kaay *et al.*, 1999). This suggests that either osmotic stress inhibits the phosphorylation of PKB or induces its dephosphorylation. Pre-treatment with calyculin blocked the inhibitory effect of osmotic stress on the activation of PKB (Van der Kaay *et al.*, 1999). This suggests that osmotic stress inhibits PKB activity through the regulation of a phosphatase. However, treatment with calyculin alone activated PKB to the same extent as IGF-1, which supports the theory that a phosphatase activity is required for the activation of PKB. Therefore it is possible that osmotic stress regulates PKB activity through inhibiting its activation, which may be mediated in part by a phosphatase.

Ahmed *et al.* (1997) investigated the role of PKB in the survival and proliferation of BaF/3 cells in response to IL-2. They expressed a wild type IL-2Rβ and a deletion mutant ΔSIL-2Rβ in BaF/3 cells and found that PKB was activated in response to IL-2 in the IL-2Rβ expressing cells but not in the ΔS expressing cells. Previous work had demonstrated that BaF/3 cells expressing the IL-2Rβ could be grown in the long-term in IL-2 while those expressing the ΔS-IL-2Rβ mutant could not (Miyazaki *et al.*, 1995). To investigate this correlation of PKB and the survival of BaF/3 cells in IL-2 signalling they expressed two forms of constitutively active PKB in ΔS-IL-2Rβ expressing BaF/3 cells. Both myristylated PKB and the constitutively active PH domain mutant E40K slowed the rate of apoptosis induced by IL-3 and IL-2 withdrawal. Additionally, cells that survived gave rise to growth factor-independent cell lines. These results are in contrast to those that we observed when gagPKB was expressed in BaF/3 cells (Figure 5.10). Ahmed and colleagues looked at the effect of PKB variants on the survival of BaF/3 cells that were transfected with IL-2Rβ mutants, and while this receptor mutant does not enable these cells to grow in response to IL-2 in the absence of active PKB, it may couple with active PKB to drive proliferation and survival. Alternatively, it is interesting that the protection that myristylated PKB afforded from apoptosis was greater to that afforded by the E40K mutant and this correlated with a greater level of basal PKB activity in myristylated PKB cell lines than in the E40K cell lines. As gagPKB had only a low level of constitutive PKB activity perhaps the signal was not sufficient to transform these cells (Figure 5.3).

Therefore, it would be interesting express the myristylated PKB mutant under the control of tetracycline in our BaF/3 cells, to investigate its effects on the proliferation and survival. Given the success of the AAAPKB mutant (Wang *et al.*, 1999), it would also be of interest to investigate the effects of its expression in our system and to compare these to the results obtained with the cxPKB mutant.

When interpreting the results presented in this chapter on the effect of the expression of PKB variants in BaF/3 cells it must be remembered that 3 isoforms of PKB exist. It is not known which isoforms of PKB are expressed in BaF/3 cells, as initial experiments using antibodies specific to each of the α, β and γ isoforms were unsuccessful due to the quality of the antibodies available. The antibodies used in these studies were raised against PKBα and the PKB variants were also of the PKBα isoform. As such, the effects of the expression of these proteins on the activity of PKBβ and PKBγ have not been investigated. Therefore it is possible that the activity of PKBβ and PKBγ could

compensate for any changes in PKBα activity thus masking any physiological or biochemical effects. It is important to firstly determine which isoforms are expressed in these cells and secondly look at the effects of the PKB variants expression on their activity. Since the issue of which isoforms of PKB are expressed in these cells was first examined new antibodies specific to each isoforms have become available and these may prove to be of sufficient quality. Alternatively RT-PCR could be performed to determine which transcripts are expressed in BaF/3 cells.

Over recent years, a number of possible targets of PKB have been identified. The effect of gagPKB and kdPKB expression on the PKB substrate Bad was investigated, but the effect of these PKB variants on other substrates was not examined. PKB is rapidly activated by cytokine-stimulation in these cells, yet it does not appear to be influencing cell survival or proliferation. By examining the effects of PKB variant expression on such downstream targets as GSK-3, and the forkhead proteins, insights might be gained into what physiological responses are regulated by PKB in these cells.

Chapter 6
General Discussion

6.1 Discussion

The serine/threonine kinase PKB has been widely implicated as a positive regulator of growth and survival in a number of cell systems (for a review see Coffer and Woodgett, 1998; Datta *et al.*, 1999) and at the conception of this work the generally accepted view was that the activation of PKB was sufficient and necessary to drive long-term growth and survival. While PKB is able to provide a survival signal in some systems, the question of whether this is a universal phenomenon had not been addressed. Therefore, this project set out to investigate the role that PKB plays in the cytokine-mediated growth and survival of haemopoietic cells. Haemopoietic cells are dependent on specific cytokines for their continued growth and survival and upon cytokine withdrawal they die by apoptosis. Thus, they provide ideal cell models in which to investigate the effects of PKB on proliferation and survival.

The first objective of this work was to determine if PKB was activated in response to the cytokine-stimulation of responsive cells. All the cytokines examined were able to activate PKB by a PI3K-mediated pathway. However, this did not correlate with cytokine-induced proliferation and survival of these cells in all cases. This suggests that PKB activation alone is not always sufficient to provide a growth and survival signal to cells. At the time of this work, the Bcl-2 family member Bad had recently been identified as a substrate of PKB (Datta *et al.*, 1997; del Peso *et al.*, 1997). Therefore, the effects of the cytokine-induced PKB activation on Bad phosphorylation were also examined. However, the patterns of Bad hyper-phosphorylation did not always correlate with the cytokine-induced PKB activation or the survival of cells. In addition, cell type differences were observed. This evidence suggests that Bad was not vital for the cytokine-mediated survival of these cells.

These data, while not directly investigating the effects mediated by PKB, suggested that PKB activation was not absolutely required for the survival of these haemopoietic cells. This hypothesis gains further support from work with PKB variants in BaF/3 cells. Preliminary results indicate that the expression of the dominant negative PKB variant cxPKB does not affect the IL-3-induced proliferation or survival of BaF/3 cells. Conversely, the expression of the constitutively active PKB variant gagPKB does not protect cells from apoptosis induced by IL-3 withdrawal, nor does it lessen the requirement of IL-3 for continued proliferation.

Over recent years, other groups have also begun to question the absolute requirement of PKB for the growth and survival of cells. Scheid *et al.* (1999) found that in MC9 cells stimulated with GM-CSF or IL-3 Bad was phosphorylated on Ser^{112} by a MEK dependent pathway and two other residues, but not on the PKB dependent Ser^{136} site. This suggests that PKB may not always be required for the hyper-phosphorylation of Bad, and preliminary results looking at the migration patterns of Bad in BaF/3 cells indicate that Bad migration patterns are not affected by the expression of dominant negative or constitutively active PKB variants (Figures 5.8 and 5.9, C. Beck and M.J. Welham unpublished data). In addition, Bad hyper-phosphorylation did not correlate with PKB activity in all cases. Collectively these data question the necessity of PKB activation for Bad hyper-phosphorylation.

Other groups have found that PKB activation is not necessary for the growth factor-induced cell survival. Uchida *et al.* (2000) found that PKB activation in the absence of PI3K activity was insufficient to promote the insulin-mediated survival of 32D cells transfected with the insulin receptor and IRS proteins. The lack of importance of PKB in cytokine-mediated survival is not limited to haemopoietic cells. EGF can protect LNCaP prostate cancer cells, in which PTEN is inactivated, from apoptosis even in the presence of LY294002 and subsequent inactivation of PI3K and PKB (Carson *et al.* 1999).

Collectively, these results have challenged the dogma that PKB is sufficient and necessary for cell survival and has tempered the current understanding of the role that PKB plays in growth and survival. It now appears that PKB is important for the survival and proliferation of some cells in response to some stimuli but in other systems other pathways are more important.

In this work PI3K and PKB were not found to be important for the IL-3-mediated survival of BaF/3 cells. This raises the question of what is required to drive proliferation and survival of these cells in response to IL-3. IL-3 binding to its receptor results in the activation of a number of pathways in addition to PI3K, including the Jak2/STAT5 and Ras/MAPK pathways. In studies where the $h\beta_c$ is expressed in BaF/3 cells, two distinct regions of the receptor that are involved in transducing growth and survival signals into the cell have been identified (Quelle *et al.*, 1994). One is dependent on the membrane proximal region of the receptor (up to residue 544) and the second is dependent on the membrane distal region.

The membrane proximal region of β_c is required for its interation with Jak2 and the subsequent activation of STAT5 (Quelle *et al.*, 1994). The activation of this pathway may be responsible for the growth and survival signal generated by this region, as stable BaF/3 transfectants expressing a constitutively active STAT5, 1*6, could be maintained for long periods in the absence of IL-3 (Nosaka *et al.*, 1999). This suggests that the activation of STAT5 is sufficient to drive the growth and proliferation of BaF/3 cells. Active STAT5 up-regulates the expression of a number of proteins, including the anti-apoptotic Bcl-2 family member Bcl-$_{XL}$ and this correlates with an increased survival of BaF/3 cells.

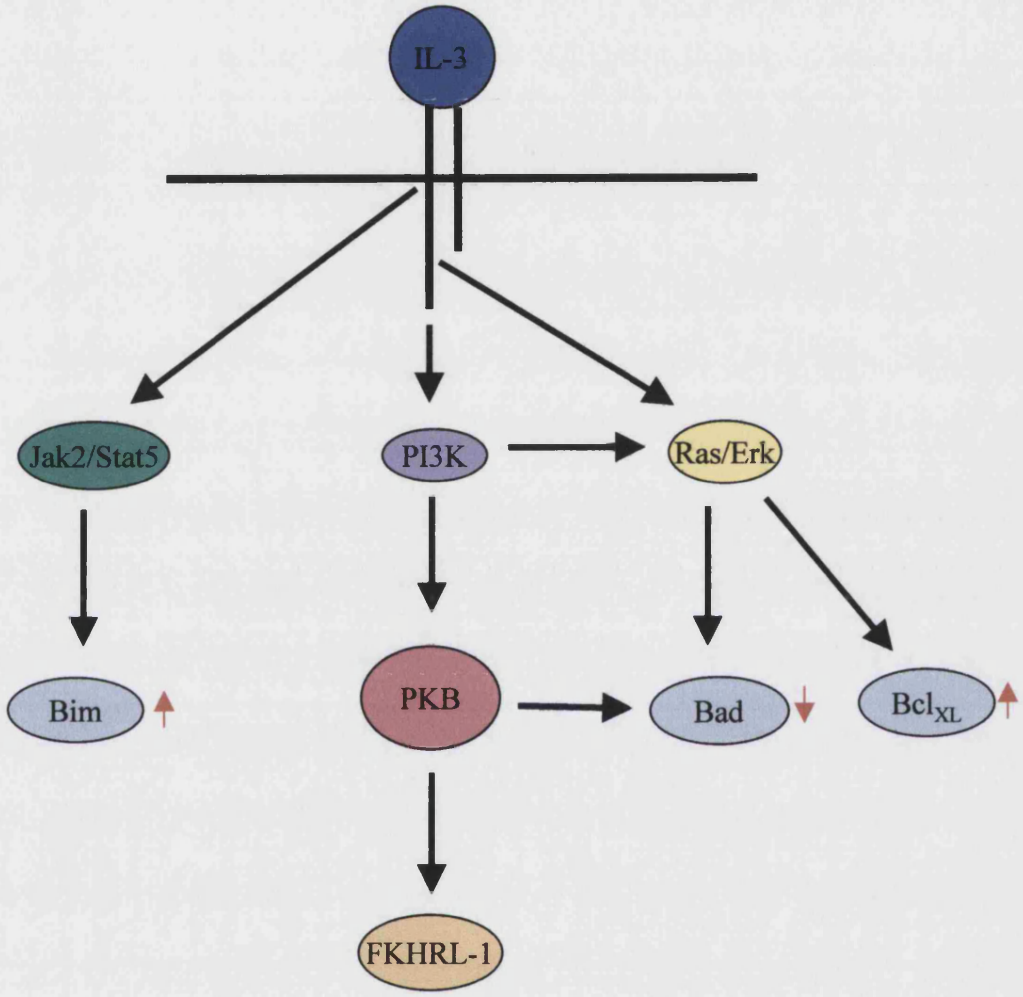
Interestingly, Santos *et al.* (2000) have recently reported that STAT5 and PI3K cooperate with each other in order to promote the survival and proliferation of BaF/3 cells. The expression of a dominant negative STAT5 (STAT5AD749) in BaF/3 cells increased cell death upon IL-3 withdrawal and this effect was enhanced when PI3K activity was inhibited by the addition of LY294002. It is interesting to note that PKB phosphorylation in these STAT5AΔ749 cells was significantly upregulated and prolonged upon stimulation with IL-3. The results in this thesis suggest that the PI3K/PKB can be dispensable for the IL-3 mediated survival of BaF/3 cells but perhaps this pathway can act in conjunction with STAT5A to help promote cell survival. It is possible that when a cell is compromised in another way, it is important to have a functional PKB to aid survival.

The distal region of β_c can also provide a survival signal to BaF/3 cells as when this region is truncated, cells undergo apoptosis in the presence of cytokines (Kinoshita *et al.*, 1995). The activation of PI3K by IL-3 requires the phosphorylation of Tyr^{577} and Tyr^{612} in the distal region of β_c (Dijkers *et al.*, 1999). However, PI3K activation alone cannot account for the survival signal that stems from the distal region of β_c as inhibition of PI3K alone does not significantly increase the apoptosis of BaF/3 cells (Craddock *et al.*, 1999; Santos *et al.*, 2000). The Ras/MAPK pathway is also dependent on this region of the receptor for activation (Kinoshita *et al.*, 1995; Wang *et al.*, 1992; Jiang *et al.*, 2000) and the expression of a constitutively active Ras mutant in the truncated β_c BaF/3 cells was sufficient to complement the defective signalling and in concert with cytokine supported the long-term growth and survival (Kinoshita *et al.*, 1995).

Recently Shinjyo *et al.* (2000) reported that IL-3-withdrawal induced the up-regulation of the BH3-only pro-apoptotic Bcl-2 family member Bim. Enforced expression of Bim to the levels seen in response to IL-3 withdrawal resulted in apoptosis even in the presence of IL-3 (Shinjyo *et al.* 2000). Expression of the dominant negative Ras, Ras (S17N), or inhibition of PI3K with wortmannin did not upregulate Bim levels. However, the inhibition of both Ras and PI3K resulted in the up-regulation of Bim and this correlated with a marked decrease in viability. (Shinjyo *et al.*, 2000). Again these results suggest that PI3K can act in conjunction with other signalling pathways to promote the survival of BaF/3 cells. PKB, however, does not appear to be involved in this pathway as expression of a dominant negative PKB, AAAPKB, in conjunction with the Ras mutant did not affect cell viability (Shinjyo *et al.*, 2000).

Collectively, these data suggest that the Jak2/STAT5, Ras/MAPK and PI3K pathways work together to promote the long-term proliferation and survival of BaF/3 cells (Figure 6.1). It is interesting that in isolation their effects on apoptosis are limited but they may co-ordinate with each other and collectively promote the survival of these cells. This is a point that needs further investigation and the tetracyline-regulated inducible expression system used in these studies would provide an ideal system in which to investigate this. The transfection of STAT5 and Ras mutants into the Δp85 cells could be used to investigate the effects of the inhibition and constitutive activation of either STAT5 or Ras in conjunction with PI3K on the proliferation and survival of BaF/3 cells.

Figure 6.1
IL-3-Induced Survival of BaF/3 Cells



Possible signalling pathways involved in the IL-3-induced survival of BaF/3 cells. IL-3 binding to its receptor on the cell membrane results in the activation of the Jak/STAT, Ras/Erk and PI3K signalling pathways. While the PI3K pathway in isolation does not appear to be involved in cell survival it has been reported to act in concert with both the Jak/STAT and Ras/Erk pathways to promote survival of BaF/3 cells. It is possible that these pathways regulate survival in part by controlling the levels and activity of Bcl2 family members Bad, Bim and Bcl_{XL}. Red arrows indicate either changes in expression or "activity" of these proteins.

The data presented in this thesis and that discussed above argue against a role for PKB in the prevention of apoptosis and proliferation in these cells. However, PKB is activated in response to IL-3 in BaF/3 cells and additionally it was activated by all the cytokines in all cell types examined. This raises a number of questions: why is PKB activated in these cells, what processes is it involved with and what is it phosphorylating?

A number of potential substrates of PKB have been identifies in recent years and in this work the phosphorylation of Bad by PKB was examined. However, the evidence presented here, while not conclusive, does not favour a role for PKB in the IL-3-mediated phosphorylation of BaF/3 cells. PKB has also been reported to phosphorylate GSK3α and β on $Ser^{21/9}$ respectively (Cross *et al.*, 1994), and the expression of cxPKB in BaF/3 cells reduced the IL-3-mediated phosphorylation of Ser^{9} on GSK3β (C. Beck and M.J. Welham, unpublished data). The forkhead family member, FKHRL-1, has also been reported to be phosphorylated in response to IL-3 stimulation in BaF/3 cells (Mathieu *et al.*, 2001). Phosphorylation on Ser^{253} is constitutive, while phosphorylation of Thr^{32} is dependent on a PI3K mediated pathway and this may well be mediated by PKB (Mathieu *et al.*, 2001).

Further insights into the role(s) that PKB plays in these and other cells could be obtained by the identification of new downstream targets of PKB. To this end gene array technology could be used to identify novel transcripts whose expression is regulated by PKB.

The long awaited PKB knockouts should also provide clues into the functions of PKB. After the submission of this thesis, work was published describing a PKBβ knock out mouse, which implicates PKBβ in the maintenance of normal glucose homeostasis (Cho *et al.*, 2002). Insulin-dependant blood glucose uptake into hormone responsive tissues was impaired leading to increase blood glucose levels and an increase production of insulin to compensate. This suggests that PKBβ is important for insulin responses. The production of knock out for PKBα and γ should give insights into where these isoforms are most important and what processes they control.

The results presented in this thesis have challenged our understanding of how cells regulate their proliferation and survival. At the conception of this work, it was generally considered that the activation of PKB was sufficient and necessary for the survival of cells. However, this work, and the work of others (Scheid *et al.*, 1999; Carson *et al.*, 1999; Shinjyo *et al.*, 2000; Uchida *et al.*, 2000), have questioned whether cells are

absolutely dependent on PKB for their continued growth and survival. Indeed the results presented here indicate that PKB is dispensable for cellular survival in some cases.

The question that now needs to be addressed is whether PKB can act in concert with other pathways to promote survival. Is PKB important for cell survival and proliferation when other pathways are compromised? Given their importance to the cell, it seems logical to have more than one pathway by which growth and survival can be regulated.

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